

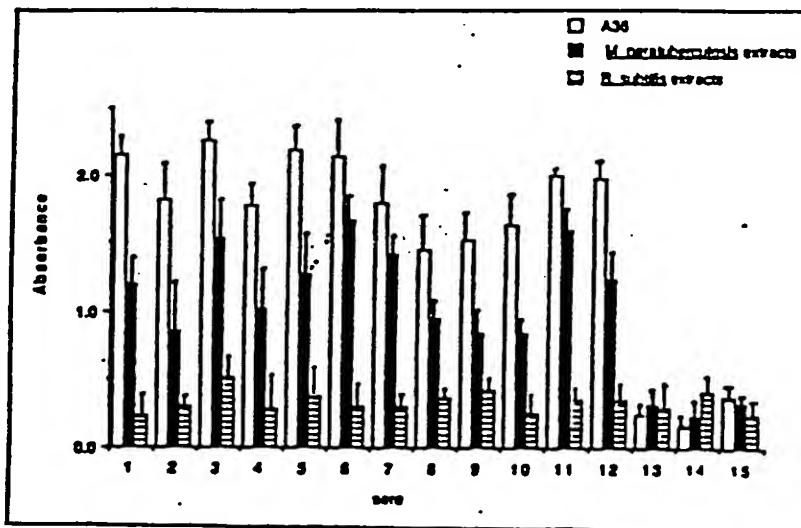


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## (54) Title: POLYPEPTIDES FROM MYCOBACTERIUM PARATUBERCULOSIS



## (57) Abstract

The invention relates to a polypeptide containing in its polypeptidic chain: the amino acid sequence of 101 amino acids of Figure 8, or a fragment of this sequence, this fragment being such that it is liable to be recognized by antibodies also recognizing the abovesaid sequence of 101 amino acids, but it is not recognized by antibodies respectively raised against *M. bovis*, *M. avium*, *M. phlei* and *M. tuberculosis*, and possibly against *M. leprae*, *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, *M. gordonaiae* and *M. smegmatis*; it is liable to generate antibodies which also recognize the abovesaid sequence of 101 amino acids but which do not recognize *M. bovis*, *M. avium*, *M. phlei* and *M. tuberculosis*, and possibly *M. leprae*, *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, *M. gordonaiae* and *M. smegmatis*; it reacts with the majority of sera from cattle suffering from Johne's disease; or the polypeptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the above-mentioned properties.

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## POLYPEPTIDES FROM MICROBACTERIUM PARATUBERCULOSIS

The invention relates to polypeptides and peptides, particularly recombinant ones, which can be used for the diagnosis of paratuberculosis in cattle and possibly of Crohn's disease in human beings. The invention also relates to a process for preparing the above-said polypeptides and peptides, which are in a state of biological purity such that they can be used as part of the active principle in the preparation of vaccines against paratuberculosis.

It also relates to nucleic acids coding for said polypeptides and peptides.

Furthermore, the invention relates to the in vitro diagnostic methods and kits using the above-said polypeptides and peptides and to the vaccines containing the above-said polypeptides and peptides as active principle against paratuberculosis.

By "recombinant polypeptides or peptides" it is to be understood that it relates to any molecule having a polypeptidic chain liable to be produced by genetic engineering, through transcription and translation, of a corresponding DNA sequence under the control of appropriate regulation elements within an efficient cellular host. Consequently, the expression "recombinant polypeptides" such as is used herein does not exclude the possibility for the polypeptides to comprise other groups, such as glycosylated groups.

The term "recombinant" indeed involves the fact that the polypeptide has been produced by genetic engineering, particularly because it results from the expression in a cellular host of the corresponding

nucleic acid sequences which have previously been introduced into an expression vector used in said host.

Nevertheless, it must be understood that the polypeptides or the peptides of the invention can be produced by a different process, for instance by classical chemical synthesis according to methods used in the protein synthesis or by proteolytic cleavage of larger molecules.

The expression "biologically pure" or "biological purity" means on the one hand a grade of purity such that the polypeptides can be used for the production of vaccinating compositions and on the other hand the absence of contaminants, more particularly of natural contaminants.

Paratuberculosis (Johne's disease) has been described as one of the most serious diseases affecting the world cattle industry. This mycobacteriosis produced by M. paratuberculosis is characterized by an ileocoecal enteritis leading successively to emaciation, dysentery, cachexy and death (Chiodini R.J. et al., 1984, "Ruminant paratuberculosis (Johne's disease): the current status and future prospects", Cronell Vet. 74:218-262). Histological examination shows oedema, infiltration and thickening of the ileal mucosa, and hypertrophy and necrosis of intestinal lymphnodes. A miliary syndrome with diffused parenchima granuloma in liver, spleen and lungs is not infrequent. The high contagiousness of this disease is due to excretion of large numbers of bacteria from the intestinal tract: contaminated pastures propagate the infection, rapidly producing live-stocks wherein infected animals represent a large part of the population. Chronical dysentery is an advanced stage of the disease, for epidemiological data suggest that the subclinical cases, with little sign of intestinal alteration correspond to the majority of infected

animals and frequently to a large proportion of a live-stock population.

Diagnosis of paratuberculosis is essential, especially in the absence of clinical symptoms: it leads to identification of hidden bacterial shedders and avoids propagation of infection. Unfortunately, diagnostic indicators for early stages of the disease are missing. In fact, identification of the etiological agent (a slow grower) is a lengthy process, and histological examination of biopsy material is difficult and expensive. More interesting appear to be the immunological procedures for analysis of humoral immune reactions (Brugère-Picoux J., 1987, "Le diagnostic de la paratuberculose chez les ruminants", Rec. Méd. Vét. 163:539-546 ; Colgrave J.S. et al., 1989, "Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture", Veterinary Microbiology 19:183-187). Although complement fixation and hemagglutination tests apparently lack both sensitivity and specificity, immunoenzymometric methods for evaluation of antimycobacterial antibodies seem to be more promising (Abbas B. et al., 1983, "Isolation of specific peptides from Mycobacterium paratuberculosis protoplasm and their use in an enzyme linked immunosorbent assay for the detection of paratuberculosis (Johne's disease) in cattle", Am. J. Vet. Res. 44:2229-2236 ; Colgrave J.S. et al., 1989, "Paratuberculosis in cattle: a comparison of three serologic tests with results of fecal culture" Veterinary Microbiology, 19:183-187 ; Yokomizo Y. et al., 1983, "Enzyme-linked immunosorbent assay for detection of bovine immunoglobulin G1 antibody to a protoplasmic antigen of Mycobacterium paratuberculosis" Am. J. Vet. Res. 44:2205-2207 ; Yokomizo Y. et al., 1985, "A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the

diagnosis of bovine paratuberculosis" Japan, J. Vet. Sci. 47:111-119).

Moreover, since slaughtering of cattle affected by tuberculosis (caused by M. bovis and/or M. tuberculosis), but not of those with paratuberculosis, is compulsory in Occidental countries, a distinction at the immunological level between the two mycobacterial diseases is essential. Moreover, M. paratuberculosis is known to be genetically close-related to M. avium (Chiodini R.J. et al., 1989, "The genetic relationship between Mycobacterium paratuberculosis and the M. avium complex" Acta Leprol. 7:249-251 ; Hurley S.S. et al., 1988, "Deoxyribonucleic acid-relatedness of Mycobacterium paratuberculosis to others members of the family Mycobacteriaceae" Int. J. Syst. Bacteriol. 38:143-146), which is a possible host of the intestinal tract of ruminants.

Taking into account the cross reactivity between M. paratuberculosis and many other mycobacteria, it was a priori a difficult approach to find an antigen containing specific epitopes liable to be used as reagents for the diagnosis of paratuberculosis, said reagents having no cross reactivity with other close related mycobacteria.

In addition to the above-mentioned aspects relative to paratuberculosis in cattle, M. paratuberculosis has been found to play an etiologic role in at least some cases of Crohn's disease in human.

The disease originally described by Crohn and coworkers was a chronic ileitis producing hyperplastic granulomata of the intestine and lymphnodes. The syndrome presently known as Crohn's disease entails inflammatory alterations of different organs of the digestive tract (mouth, larynx, esophagus, stomach, ileum and colon). Segments of the

motive apparatus (joints, muscles and bones) can also be involved. Isolation of mycobacteria from patients affected by the Crohn's disease has been repeatedly related: in several instances isolates were identified as M. paratuberculosis. The induction by these isolates of a syndrome mimicking Crohn's disease in laboratory animals and primates has been successful. In a recent review article (Chiodini R.J., 1989, "Crohn's disease and the mycobacterioses: a review and comparison of two disease entities", Clin. Microbiol. Rev. 2:90-117), Chiodini suggests this syndrome to be the expression of several pathological entities and concludes, that, if Crohn's disease has a mycobacterial etiology, the most likely agent would be M. paratuberculosis.

At this present time, larger epidemiological investigation with an ELISA based on a specific protein of M. paratuberculosis is expected to help to solve the problem of the etiology of this enteritis resembling in many respects the Johne's disease of cattle.

The expression "cattle" means ruminants, such as bovines, sheeps, goats, cervidae, but also include some non ruminant animals which may also be infected by Johne's disease such as monkeys and horses.

An aspect of the invention is to provide recombinant polypeptides which can be used as purified antigens for the detection and control of paratuberculosis.

Another aspect of the invention is to provide nucleic acids coding for the peptidic chains of biologically pure recombinant polypeptides which enable their preparation on a large scale.

Another aspect of the invention is to provide antigens which can be used in serological tests as an in vitro rapid diagnosis of paratuberculosis, as well as in skin tests for in vivo diagnosis of

paratuberculosis and as an immunogenic principle in vaccines.

Another aspect of the invention is to provide a rapid in vitro diagnostic means for paratuberculosis, enabling it to discriminate between cattle suffering from tuberculosis from the ones suffering from paratuberculosis.

Another aspect of the invention is to provide a rapid in vitro diagnostic means for paratuberculosis, enabling it to discriminate between cattle suffering from paratuberculosis from the ones infected or colonized by M. avium, M. bovis or M. tuberculosis or M. phlei.

Another aspect of the invention is to provide in vitro diagnostic means for patients suffering from Crohn's disease.

The invention relates to an antigen complex from M. paratuberculosis, named hereafter "the antigen A36", liable to be obtained as follows:

- sonication of bacterial suspensions of M. paratuberculosis to obtain a homogenate (also named sonicate),
- centrifugation of the above-mentioned homogenate to obtain a supernatant (which corresponds to the cytoplasm of the bacteria),
- RNAase digestion of the above-mentioned supernatant,
- fractionation of the digested supernatant, for instance by gel exclusion chromatography, for instance on Sepharose 6B columns,
- recovery of the antigen complex (A36) which is the excluded fraction of the fractionation.

It is to be noted that the antigen complex hereabove defined corresponds to the TMA complex (thermostable macromolecular antigens), belonging to a family of complexes present in all mycobacteria and

consisting of or containing lipid, polysaccharide and protein moieties.

The proteic part of the antigen complex of the invention can be fractionated and visualized as follows:

- fractionation of the proteins of the above-mentioned antigen complex by electrophoresis in a gel, for instance 10% polyacrylamide gels to obtain the protein on bands,
- detection of the proteins by staining for instance with Coomassie blue.

The polypeptides of the invention contain in their polypeptidic chain:

- the amino acid sequence of 101 amino acids of Figure 8,
- or a fragment of this sequence, this fragment being such that:
  - . it is liable to be recognized by antibodies also recognizing the abovesaid sequence of 101 amino acids, but it is not recognized by antibodies raised respectively against M. bovis, M. avium, M. phlei and M. tuberculosis,
  - . it is liable to generate antibodies which also recognize the abovesaid sequence of 101 amino acids but which do not recognize M. bovis, M. avium, M. phlei and M. tuberculosis,
  - . it reacts with the majority of sera from cattle suffering from Johnes's disease,
- or the polypeptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the above-mentioned properties.

Recognition of one of the above-mentioned fragments by the above-mentioned antibodies - or of the abovesaid sequence of 101 amino acids by the above-

mentioned antibodies - means that the above-mentioned fragment can form a complex with one of the above-said antibodies.

The formation of the complex antigen (i.e. the sequence of 101 amino acids or of the above-said fragment) - antibody and the detection of the existence of a formed complex can be done according to classical techniques such as the ones using a marker labeled by radioactive isotopes or by an enzyme.

Hereafter is also given in a non limitative way, a test for giving evidence of the fact that polypeptides of the invention are recognized selectively by the majority of the sera from cattle suffering from Johne's disease (immunodominant polypeptides), for instance bovines.

This test is an immunoblotting (Western blotting) analysis, in the case where the polypeptides of the invention are obtained by recombinant techniques. This test can also be used for polypeptides of the invention obtained by a different preparation process. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polypeptides of the invention are blotted onto nitrocellulose membranes (Hybond C. (Amersham)) as described by Towbin H. et al., 1979, "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications", Proc. Natl. Acad. Sci. USA 76:4350-4354. The expression of polypeptides of the invention fused to  $\beta$ -galactosidase in E. coli Y1089, is visualized by the binding of a polyclonal rabbit anti-A36 antiserum (or polyclonal rabbit anti-homogenate antiserum defined hereafter in the examples, or polyclonal rabbit anti- $\beta$ gal-p362 antiserum, defined hereafter in the examples) (1:1,000) or by using a monoclonal anti- $\beta$ -galactosidase antibody (Promega). The secondary antibody (anti-rabbit immunoglobulin G and

anti-mouse immunoglobulin G respectively, both alkaline phosphatase conjugated) is diluted as recommended by the supplier (Promega). Colour reaction is developed by adding NBT/BCIP (Nitro Blue Tetrazolium 5-bromo 4-chloro-3-indolyl phosphate [Promega]) using conditions recommended by suppliers.

In order to identify selective recognition of polypeptides of the invention and of fusion proteins of the invention by sera of bovine suffering from Johne's disease, nitrocellulose sheets are incubated overnight with each of these sera (1:50) (after blocking aspecific protein-binding sites).

Reactive areas on the nitrocellulose sheets are revealed by incubation with peroxidase conjugated goat anti-bovine immunoglobulin G antibody (Dakopatts, Copenhagen, Denmark) (1:200) for 4h, and after repeated washings, color reaction is developed by adding α-chloronaphthol (Bio-Rad Laboratories, Richmond, Calif.) in the presence of hydrogen peroxide.

The non-recognition of the antibodies raised against the above-mentioned fragments of the invention by M. bovis, M. avium, M. phlei and M. tuberculosis and by other mycobacteria can be done according to a process detailed in the examples.

As to the non-recognition of the above-mentioned fragments of the invention by antibodies raised respectively against M. bovis, M. avium, M. phlei and M. tuberculosis or other mycobacteria, it can also be done according to a process detailed in the examples.

Advantageous above-defined fragments of the invention are liable not to be recognized by antibodies raised against other mycobacteria such as M. leprae, M. intracellulare, M. scrofulaceum, M. fortuitum, M. gordonae and M. smegmatis, and are liable to generate antibodies which do not recognize M. leprae, M.

intracellulare, M. scrofulaceum, M. fortuitum, M. gordonae and M. smegmatis.

It goes without saying that the free reactive functions which are present in some of the amino acids, which are part of the constitution of the polypeptides of the invention, particularly the free carboxyl groups which are carried by the groups Glu and Asp or by the C-terminal amino acid on the one hand and/or the free NH<sub>2</sub> groups carried by the N-terminal amino acid or by amino acids inside the peptidic chain, for instance Lys, on the other hand, can be modified in so far as this modification does not alter the above mentioned properties of the polypeptide.

The molecules which are thus modified are naturally part of the invention. The above mentioned carboxyl groups can be acylated or esterified.

Other modifications are also part of the invention. Particularly, the amine or carboxyl functions or both of terminal amino acids can be themselves involved in the bond with other amino acids. For instance, the N-terminal amino acid can be linked to the C-terminal amino acid of another peptide comprising from 1 to several amino acids.

Furthermore, any peptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids of the polypeptides according to the invention are part of the invention in so far as this modification does not alter the above mentioned properties of said polypeptides.

The polypeptides according to the invention can be glycosylated or not, particularly in some of their glycosylation sites of the type Asn-X-Ser or Asn-X-Thr, X representing any amino acid.

An advantageous recombinant polypeptide of the invention is constituted by the sequence represented on Figure 8, extending from the extremity constituted by

amino acid at position (1) to the extremity constituted by amino acid at position (101), or by the following peptides:

Glu-Phe-Pro-Gly-Gly-Gln-Gln-His-Ser-Pro-Gln,

(position 1 to 11 on Figure 8)

Gln-Gln-Ser-Tyr-Gly-Gln-Glu-Pro-Ser-Ser-Pro-Ser-Gly-  
Pro-Thr-Pro-Ala

(position 85 to 101 on Figure 8).

It is to be noted that this polypeptide is derived from the expression product of a DNA derived from the nucleotide sequence coding for a polypeptide of 10 kDa being the carboxy terminal part of a 34 kDa protein of M. paratuberculosis, defined hereafter.

An advantageous recombinant polypeptide of the invention is characterized by the fact that:

- it contains the amino sequence of 101 amino acids of Figure 8 as its C-terminal part,
- it has a molecular weight of about 34kDa, in SDS-PAGE,
- it is coded by a nucleotide sequence liable to hybridize with the complementary strand of the sequence of Figure 11,
- it reacts with the majority of sera from cattle suffering from Johne's disease,
- it is advantageously liable to elicit a cellular immune response in sensitized subjects.

Subjects can be either test animals such as mice or guinea pigs or cattle or human beings.

"Sensitized" means that these subjects have been in contact previously with M. paratuberculosis, resulting in a priming of the cellular immune system.

Sensitization can be induced by inoculating the subjects with killed or attenuated M. paratuberculosis or it can result from a natural infection with M. paratuberculosis.

A positive cellular immune response to the polypeptides of the invention can be detected for example in vivo by a delayed - type hypersensitivity reaction upon skintesting with the polypeptides of the invention or in vitro by proliferation of peripheral blood lymphocytes isolated from sensitized subjects, in response to the added polypeptides.

An advantageous recombinant polypeptide of the invention contains or is constituted by the amino acid sequence of Figure 11.

Another advantageous recombinant polypeptide of the invention contains or is constituted by the amino acid sequence extending from amino acid at position (1) to the amino acid at position (199), of Figure 11.

It is to be noted that this polypeptide is a 34 kDa protein which is present in the proteic part of the TMA complex of M. paratuberculosis (A36).

Hereafter is given, in a non limitative way, a process for preparing this 34 kDa protein of the invention.

The DNA sequence (306 bp) coding for p362, being the carboxyterminal end of the 34 kDa protein has been determined (see Figure 8). It contains a unique ApaI (GGGCC) site at position 141.

Using this information, the full gene coding for the 34 kDa protein can be isolated as follows:

An oligonucleotide coding for a stretch of at least 30 bp, situated within the region EcoRI-ApaI (1-141 bp) of the known sequence, is synthesized.

It is labeled and used as a probe to hybridize to the DNA of M. paratuberculosis (strain ATCC 19698), which has previously been cut by ApaI, separated by agarose gel electrophoresis, denatured and transferred to a nylon membrane.

This hybridization indicates a band on the nylon membrane of around 1500 bp, which contains the coding

part for the rest of the 34 kDa protein. After having located this 1500 bp fragment, flanked by 2 ApaI sites, in the agarose gel, it is isolated from the gel, purified and subcloned in the ApaI site of the sequencing vector pBluescript SK'.

After sequencing of this fragment, the coding region, starting with the initiation codon ATG or GTG, is delineated. Using a restriction site near the initiation codon (5' end), naturally present or created by site-directed mutagenesis, and the ApaI site at the 3' end, the DNA fragment coding for the N-terminal part of the protein (about 750 bp) is excised from pBluescript SK', and purified. It is ligated to the ApaI site of the fragment coding for the C-terminal part of p362 (142-306, Figure 8), that for example has been prepared synthetically.

The complete gene coding for the 34 kDa protein (about 910 bp) is subcloned in an expression vector and expressed in E. coli. The recombinant 34 kDa protein is then purified.

The invention also relates to the amino acid sequences constituted by the above mentioned polypeptides and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising for instance from about 1 to about 1100 amino acids. These amino acid sequences will be called fusion proteins.

In an advantageous fusion protein of the invention, the heterologous protein is  $\beta$ -galactosidase.

The invention also relates to a nucleic acid characterized by the fact that it comprises or is constituted by:

- a nucleotide chain liable to hybridize with the nucleotide chain coding for the polypeptides according to the invention, or

- a nucleotide chain coding for the polypeptides according to the invention, or
- the complementary sequences of the above nucleotide chains.

The invention also relates to nucleic acids comprising nucleotide sequences which hybridize with the nucleotide sequences coding for any of the above mentioned polypeptides under the following hybridization conditions:

- hybridization and wash medium:

- \* a preferred hybridization medium contains about 3 x SSC [SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7], about 25 mM of phosphate buffer pH 7.1, and 20% deionized formamide, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone and about 0.1 mg/ml sheared denatured salmon sperm DNA,

- \* a preferred wash medium contains about 3 x SSC, about 25 mM phosphate buffer, pH 7.1 and 20% deionized formamide;

- hybridization temperature (HT) and wash temperature (WT) for the nucleic acids of the invention defined by x-y: i.e. by the sequence extending from the extremity constituted by the nucleotide at position (x) to the extremity constituted by the nucleotide at position (y) represented on Figures 7A, 7B or 7C:

1 - 306 (for Figures 7B and 7C) or

HT = WT = 65°C

1 - 307 (for Figure 7A)

1 - 507 (for Figures 7B and 7C)

HT = WT = 65°C

1 - 508 (for Figure 7A)

The above mentioned temperatures are to be considered as approximately  $\pm 5^\circ\text{C}$ .

It is to be noted that in the above defined nucleic acids, as well as in the hereafter defined

nucleic acids, the nucleotide sequences which are brought into play are such that T can be replaced by U.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (307) represented in Figure 7A,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (508) represented in Figure 7A, wherein
  - X and E represent phosphodiester bonds,
  - Y and F represent respectively G and C,
  - Z and H represent respectively C and G,  
or
  - X and E represent respectively G and C,
  - Y and F represent respectively C and G,
  - Z and H represent phosphodiester bonds.

A group of preferred nucleic acids of the invention comprises one at least of the following nucleotide sequences:

- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (306) represented in Figure 7B,
- the one extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (507) represented in Figure 7B.

The nucleotide sequence represented in Figure 7B corresponds to the one represented in Figure 7A, wherein

- X and E represent phosphodiester bonds,
- Y and F represent respectively G and C,

- Z and H represent respectively C and G.

The invention also relates to a nucleic acid characterized by the fact that it comprises or is constituted by a nucleotide chain,

- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (306) on Figure 7C, or
- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (507) on Figure 7C.

The nucleotide sequence represented on Figure 7C corresponds to the one represented on Figure 7A, wherein

- X and E represent respectively G and C,
- Y and F represent respectively C and G,
- Z and H represent phosphodiester bonds.

The invention also relates to a nucleic acid which comprises or is constituted by:

- a nucleotide sequence liable to hybridize with the complementary strand of the nucleotide sequence of Figure 11, or with the complementary strand of the nucleotide sequence extending from nucleotide at position (742) to nucleotide at position (1338) of Figure 11,
- the nucleotide sequence of Figure 11 or the nucleotide sequence extending from nucleotide at position (742) to nucleotide at position (1338) of Figure 11,
- the complementary sequences of the nucleotide sequences above-defined.

From the nucleic acids of the invention, probes (i.e. cloned or synthetic oligonucleotides) can be inferred.

These probes can be from 15 to the maximum number of nucleotides of the selected nucleic acids. The oligonucleotides can also be used either as

amplification primers in the PCR technique (PCR, Mullis and Faloona, Methods in Enzymology, vol. 155, p. 335, 1987) to generate specific enzymatically amplified fragments and/or as probes to detect fragments amplified between bracketing oligonucleotide primers.

The specificity of a PCR-assisted hybridization assay can be controlled at different levels.

The amplification process or the detection process or both can be specific. The latter case giving the higher specificity is preferred.

The invention also relates to any recombinant nucleic acid containing at least one of the nucleic acids of the invention combined to or inserted in a heterologous nucleic acid.

The invention relates more particularly to recombinant nucleic acid such as defined, in which the nucleotide sequence of the invention is preceded by a promoter (particularly an inducible promoter) under the control of which the transcription of said sequence is liable to be processed and possibly followed by a sequence coding for transcription termination signals.

The invention also relates to the recombinant nucleic acids in which the nucleic acid sequences coding for the polypeptide of the invention and possibly the signal peptide, are recombined with control elements which are heterologous with respect to the ones to which they are normally associated with in the mycobacterial genome and, more particularly, the regulation elements adapted to control their expression in the cellular host which has been chosen for their production.

The invention also relates to recombinant vectors, particularly for cloning and/or expression, comprising a vector sequence, notably of the type plasmid, cosmid or phage or virus DNA, and a recombinant nucleic acid

of the invention, inserted in one of the non essential sites for its replication.

According to an advantageous embodiment of the invention, the recombinant vector contains necessary elements to promote the expression in a cellular host of polypeptides coded by nucleic acids according to the invention inserted in said vector and notably a promoter recognized by the RNA polymerase of the cellular host, particularly an inducible promoter and possibly a sequence coding for transcription termination signals and possibly a signal sequence and/or an anchoring sequence.

According to another additional embodiment of the invention, the recombinant vector contains the elements enabling the expression by E. coli of a fusion protein consisting of the polypeptide of  $\beta$ -galactosidase or part thereof linked to a polypeptide coded by a nucleic acid according to the invention.

The invention also relates to a cellular host, chosen from among bacteria such as E. coli or chosen from among eukaryotic organism, such as CHO cells or insect cells, which is transformed by a recombinant vector according to the invention, and containing the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to the invention in this host.

The invention relates to an expression product of a nucleic acid expressed by a transformed cellular host according to the invention.

The invention also relates to a process for preparing a recombinant polypeptide according to the invention comprising the following steps:

- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to the invention,

- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium, or from the cellular host, and
- possibly the purification of the polypeptide produced, eventually by means of immobilized metal ion affinity chromatography (IMAC).

The polypeptides of the invention can be prepared according to the classical techniques in the field of peptide synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book titled "Methode der organischen Chemie" (Method of organic chemistry) edited by E. Wunsh, vol. 15-I et II. THIEME, Stuttgart 1974.

The polypeptides of the invention can also be prepared in solid phase according to the method described by Atherton & Shepard in their book titled "Solid phase peptide synthesis" (Ed. IRL Press, Oxford, NY, Tokyo, 1989).

The invention also relates to a process for preparing the nucleic acids according to the invention.

A suitable method for chemically preparing the single-stranded nucleic acids (containing at most 100 nucleotides of the invention) comprises the following steps:

- DNA synthesis using the automatic  $\beta$ -cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986.

In the case of single-stranded DNA, the material which is obtained at the end of the DNA synthesis can be used as such.

A suitable method for chemically preparing the double-stranded nucleic acids (containing at most

100 bp of the invention) comprises the following steps:

- DNA synthesis of one sense oligonucleotide using the automatic  $\beta$ -cyanoethyl phosphoramidite method described in Bioorganic Chemistry 4; 274-325, 1986, and DNA synthesis of one anti-sense oligonucleotide using said above-mentioned automatic  $\beta$ -cyanoethyl phosphoramidite method,

- combining the sense and anti-sense oligonucleotides by hybridization in order to form a DNA duplex,

- cloning the DNA duplex obtained into a suitable plasmid vector and recovery of the DNA according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

A method for the chemical preparation of nucleic acids of length greater than 100 nucleotides - or bp, in the case of double-stranded nucleic acids - comprises the following steps:

- assembling of chemically synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the succession of amino acids in the natural peptide, according to the principle described in Proc. Nat. Acad. Sci. USA 80; 7461-7465, 1983,

- cloning the DNA thereby obtained into a suitable plasmid vector and recovery of the desired nucleic acid according to classical methods, such as restriction enzyme digestion and agarose gel electrophoresis.

The invention also relates to antibodies themselves formed against the polypeptides according to the invention, and characterized by the fact that they recognize neither M. bovis, nor M. avium, nor M. phlei, nor M. tuberculosis.

It goes without saying that this production is not limited to polyclonal antibodies.

It also relates to any monoclonal antibody produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat, immunized against the purified polypeptide of the invention on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by its ability to produce the monoclonal antibodies recognizing the polypeptide which has been initially used for the immunization of the animals.

The invention also relates to any antibody of the invention labeled by an appropriate label of the enzymatic, fluorescent or radioactive type.

The polypeptide which is advantageously used to produce antibodies, particularly monoclonal antibodies, is the one or part of the one extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (101) represented on Figure 8.

Variations of this peptide are also possible depending on its intended use. For example, if the peptide is to be used to raise antisera, the peptide may be synthesized with an extra cysteine residue added. This extra cysteine residue is preferably added to the amino terminus and facilitates the coupling of the peptide to a carrier protein which is necessary to render the small peptide immunogenic. If the peptide is to be labeled for use in radioimmune assays, it may be advantageous to synthesize the protein with a tyrosine attached to either the amino or carboxyl terminus to facilitate iodination. This peptide possesses therefore the primary sequence of the peptide above-mentioned but with additional amino acids which do not appear in the primary sequence of the protein and whose sole function is to confer the desired chemical properties to the peptide.

The invention also relates to a process for detecting in vitro antibodies related to paratuberculosis in a biological sample of an animal liable to contain them, this process comprising

- contacting the biological sample with a polypeptide or a peptide according to the invention, or the expression product of the invention, under conditions enabling an in vitro immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by an animal serum, and particularly by bovine serum.

The detection can be carried out according to any classical process.

By way of example a preferred method brings into play an immunoenzymatic process according to ELISA technique or immunofluorescent or radiimmunological (RIA) or the equivalent ones.

Thus the invention also relates to any polypeptide according to the invention labeled by an appropriate label of the enzymatic, fluorescent, radioactive... type.

Such a method for detecting in vitro antibodies related to paratuberculosis comprises for instance the following steps:

- deposit of determined amounts of a polypeptidic composition according to the invention in the wells of a titration microplate,
- introduction into said wells of increasing dilutions of the serum to be diagnosed,
- incubation of the microplate,
- repeated rinsing of the microplate,
- introduction into the wells of the microplate of labeled antibodies against the blood immunoglobulins,

- the labeling of these antibodies being based on the activity of an enzyme which is selected from among the ones which are able to hydrolyze a substrate by modifying the absorption of the radiation of this latter at least at a given wave length,
- detection by comparing with a control standard of the amount of hydrolyzed substrate.

The invention also relates to a process for detecting and identifying in vitro antigens of M. paratuberculosis in an animal biological sample liable to contain them, this process comprising:

- contacting the biological sample with an appropriate antibody of the invention under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. paratuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

Preferably, the biological medium is constituted by serum or faeces, milk or urine, particularly of bovine origin.

Appropriate antibodies are advantageously monoclonal antibodies directed against the above-mentioned peptide.

The invention also relates to an additional method for the in vitro diagnosis of paratuberculosis in an animal liable to be infected by Mycobacterium paratuberculosis comprising:

- contacting a biological sample taken from an animal with a polypeptide or a peptide of the invention, or the expression product of the invention, under conditions enabling an in vitro immunological reaction between said polypeptide or peptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has possibly been formed.

To carry out the in vitro diagnostic method for paratuberculosis in an animal liable to be infected by Mycobacterium paratuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- a polypeptide or a peptide according to the invention, or the expression product of the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned polypeptide or peptide is not labeled.

The invention also relates to an additional method for the in vitro diagnosis of paratuberculosis in an animal liable to be infected by M. paratuberculosis, comprising the following steps:

- contacting a biological sample of said animal with an appropriate antibody of the invention under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. paratuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

To carry out the in vitro diagnostic method for paratuberculosis in an animal liable to be infected by Mycobacterium paratuberculosis, the following necessary or kit can be used, said necessary or kit comprising:

- an antibody of the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagent possibly having a label or being liable to be recognized by a labeled reagent, more

particularly in the case where the above-mentioned antibody is not labeled.

An advantageous kit for the in vitro diagnosis of paratuberculosis comprises:

- at least a suitable solid phase system, e.g. a microtiter-plate for deposition thereon of the biological sample to be diagnosed in vitro,
- a preparation containing one of the monoclonal antibodies of the invention,
- a specific detection system for said monoclonal antibody,
- appropriate buffer solutions for carrying out the immunological reaction between a test sample and said monoclonal antibody on the one hand, and the bonded monoclonal antibodies and the detection system on the other hand.

The invention also relates to a kit, as described above, also containing a preparation of one of the polypeptides or peptides of the invention, said antigen of the invention being either a standard (for quantitative determination of the antigen of M. paratuberculosis which is sought) or a competitor, with respect to the antigen which is sought, for the kit to be used in a competition dosage process.

The invention also relates to a method for the in vitro diagnosis of Crohn's disease in a patient liable to be infected by Mycobacterium paratuberculosis comprising the following steps:

- contacting the biological sample with an appropriate antibody according to the invention, under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. paratuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

The invention also relates to a method for the in vitro diagnosis of Crohn's disease in a patient liable to be infected by M. paratuberculosis, comprising the following steps:

- contacting a biological sample taken from a patient with a polypeptide or peptide according to the invention, or the expression product of the invention, under conditions enabling an in vitro immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has been possibly formed.

The invention also relates to a necessary or kit for an in vitro diagnosis method of Crohn's disease in a patient liable to be infected by Mycobacterium paratuberculosis, said necessary or kit comprising:

- an antibody according to the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagents possibly having a label or being liable to be recognized by a labeled reagent, more particularly in the case where the above-mentioned antibody is not labeled.

The invention also relates to a necessary or kit for an in vitro diagnosis method of Crohn's disease in a patient liable to be infected by Mycobacterium paratuberculosis said necessary or kit comprising:

- a polypeptide or a peptide according to the invention, or the expression product of the invention,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or

being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned polypeptide is not labeled.

The invention also relates to an immunogenic composition comprising a polypeptide or a peptide according to the invention, or the expression product of the invention, in association with a pharmaceutically acceptable vehicle.

The invention also relates to a vaccine composition comprising among other immunogenic principles anyone of the polypeptides or peptides of the invention or the expression product of the invention, possibly coupled to a natural protein or to a synthetic polypeptide having a sufficient molecular weight so that the conjugate is able to induce in vivo the production of antibodies neutralizing Mycobacterium paratuberculosis, or induce in vivo a protective cellular immune response by activating M. paratuberculosis antigen-responsive T cells.

The invention also relates to a necessary or kit for the diagnosis of prior exposure of an animal to M. paratuberculosis, said necessary or kit containing a preparation of at least one of the polypeptides or peptides of the invention, or the expression product of the invention, with said preparation being able to induce in vivo after being intradermally injected to an animal a delayed type hypersensitivity reaction, at the site of injection, in case the animal has had prior exposure to M. paratuberculosis.

Other characteristics and advantages of the invention will appear in the following examples and the figures illustrating the invention.

#### LEGENDS TO FIGURES

- Figure 1(1) represents the two-dimensional cross immunolectrophoresis (CIE) of total cytoplasm (the

supernatant fraction obtained after centrifugation of the sonicate) from M. paratuberculosis and Figure 1(2) represents the two-dimensional cross immunoelectrophoresis of the exclusion fraction obtained by gel exclusion chromatography of the same cytoplasm.

In the second dimension (upward in the figure), migration was made in a gel containing rabbit antiserum directed against the mycobacterial sonicate. Preparations in 1 and 2 contained 10 µg of proteins. This figure identifies the TMA complex of M. paratuberculosis (A36) present in the exclusion fraction.

- Figure 2 represents the serological analysis of infected animals with polypeptide p362. Multiwell plates were coated with 4 µg of proteins/well of E. coli-a362 total cytoplasm (white) or E. coli-control total cytoplasm (black). Samples of diluted (1/400) bovine sera previously exhausted by incubation with E. coli-control homogenate (said homogenate and total cytoplasm being obtained in the same way as M. paratuberculosis homogenate and total cytoplasm as described above) were added, followed by washing, incubation with labeled anti-bovine Ig, peroxidase reagents and spectrophotometric reading at 450 nm.

The following sera were used: asymptomatic non-excretory (sample 1), asymptomatic excretory (samples 2 to 13), symptomatic excretory (samples 14 to 24) and healthy bovine (samples 26 to 32).

- Figure 3 represents the serological analysis of infected animals with a A36-based immunoassay.

Multiwell plates were coated with comparable amounts (0.5 µg total proteins/well) of: M. paratuberculosis total cytoplasm (black), A36 (white) and B. subtilis total cytoplasm (control: hatched). Samples of diluted (1/400) bovine sera previously

exhausted by incubation with B. subtilis homogenate (said homogenate and total cytoplasm being obtained in the same way as M. paratuberculosis homogenate and total cytoplasm as above-described) were added, followed by washing, incubation with labeled anti-bovine Ig, peroxidase reagents and spectrophotometric reading at 450 nm. The following bovine sera were used: a) symptomatic-excretory forms of paratuberculosis (samples 1 to 7); b) asymptomatic-excretory forms (samples 8 to 12); and c) healthy cattle (samples 13 to 15). Mean values of absorbance and standard deviations are the results of 4 repeats.

Figure 4 represents the recognition of different A36 proteins by the sera of infected bovines. A36 proteins from M. paratuberculosis were fractionated by gel electrophoresis and transferred to nitrocellulose. Membranes were incubated with sera from uninfected (lane 8) or infected animals (lanes 4 to 7), either pre-absorbed (lane 7) or not (lanes 4, 5, 6) with a mixture of homogenates of M. avium, M. bovis and M. phlei. Membrane-bound primary Ig were revealed by labeled secondary Ig. Sera of infected animals were as follows: asymptomatic-non excretory (lane 4), asymptomatic-excretory (lane 5), and symptomatic-excretory (lane 6, 7) cases of paratuberculosis. Reference molecular weight standards (lane 1) and A36 proteins (lane 2) were stained by India ink. Reference: A36 proteins immunoblotted with anti-A36 rabbit antiserum (lane 3).

Figure 5 represents the analysis of the size of the polypeptide (p362) fused to  $\beta$ -galactosidase expressed by recombinant clone a362 (hereafter defined). This fusion protein is named  $\beta$ gal-p362.

Lysate proteins of E. coli Y1089 lysogenized either by standard  $\lambda$ gt11 (tracks C and E) or by the same phage carrying the insert coding for p362 (clone

a362) (tracks D and F) were fractionated by 7.5% polyacrylamide gel electrophoresis. Tracks C and D and molecular weight standards (tracks A and B) were stained with Coomassie brilliant blue, whereas tracks E and F were treated with rabbit anti-A36 antiserum and stained with peroxidase-labeled anti-rabbit antiserum.

Figure 6 represents the evidence of the belonging of the recombinant polypeptide p362 to the 34 kd protein of the A36 complex.

The TMA complex from M. paratuberculosis was dissociated and its protein components were fractionated by 10% polyacrylamide gel electrophoresis and transblotted to a nitrocellulose membrane. Fractionated proteins were either stained with India ink (track b) or incubated with rabbit anti- $\beta$ gal-p362 antiserum (track c). Track a: molecular weight standards.

Figure 7A represents the nucleic acid sequence encompassing the nucleic acid sequence of Figure 7B and the one of Figure 7C.

Figure 7B represents a sequence homologous to the one represented on Figure 7C.

Figure 7C represents the base sequence of the M. paratuberculosis genomic fragment present in clone a362 and coding for p362.

It should be noted that the two EcoRI sites [GAATTC] present at both ends of the sequence are a result of the cloning strategy and are not naturally present in the genomic sequence.

Figure 8 represents the amino acid sequence and corresponding nucleotide sequence of the recombinant polypeptide p362.

It should be noted that the first two amino acids, corresponding to the EcoRI sites in the DNA sequence, are not naturally present in the native protein, but are a result of cloning.

Figure 9a corresponds to the restriction and genetic map of the pmTNF-MPH plasmid used in Example II for the expression of p362 of the invention in E. coli.

Figure 9b corresponds to the pmTNF-MPH nucleic acid sequence.

On this figure, the origin of nucleotide stretches used to construct plasmid pmTNF-MPH is specified hereafter.

#### Position

- 1-208: lambda PL containing EcoRI blunt-MboII blunt fragment of pPL(λ) (Pharmacia)
- 209-436: synthetic DNA fragment
- 230-232: initiation codon (ATG) of mTNF fusion protein
- 236-307: sequence encoding AA 2 to 25 of mature mouse TNF
- 308-384: multiple cloning site containing His<sub>6</sub> encoding sequence at position 315-332
- 385-436: HindIII fragment containing E. coli trp terminator
- 437-943: rxnBT<sub>1</sub>T<sub>2</sub> containing HindIII-SspI fragment from pKK223 (Pharmacia)
- 944-3474: DraI-EcoRI blunt fragment of pAT<sub>153</sub> (Bioexcellence), containing the tetracycline resistance gene and the origin of replication.

Figure 10 represents the complete amino acid sequence of the recombinant polypeptide mTNF-H6-p362. The amino acids 1-26 represent the mTNF part, the amino acids from 27-46 correspond to the polylinker part (H6) and the remaining amino acids (47-147) represent the M. paratuberculosis 10 kDa polypeptide (p362).

Figure 11 represents the DNA sequence containing the nucleic acid coding for the protein of 34 kDa hereabove defined and the corresponding amino acid sequence. Nucleotides are numbered in the right-hand

side margin and amino acids are numbered below the protein sequence.

It is to be noted that the arrow before amino acid 200 corresponds to the third amino acid of Figure 8, since the first two amino acids of Figure 8 are artificial, corresponding to the EcoRI site from cloning.

Table 5 hereafter corresponds to the complete restriction site analysis of pmTNF-MPH.

Table 5

\*\*\*\*\*  
\* RESTRICTION-SITE ANALYSIS \*  
\*\*\*\*\*

Done on DNA sequence PHTNPHII.

Total number of bases is: 3474.  
Analysis done on the complete sequence.

List of cuts by enzyme.

Acc I	:	371	2018					
Acy I	:	788	2264	2921	3035	3056		
Afl I	II	387						
Afl I	III	1698						
Aha III	:	224						
Alu I	:	386	439	1141	1398	1534	1760	2382
Alu NI	:	1289						2785
Apa I	:	345						3441
Apa LI	:	1384						3456
Asp 718I	:	210						
Rsa I	:	341	342	547	676	766	1988	2030
								2209
								2333
								2582
								267
Ava I	:	2945	3297					
Ava II	:	338	2043					
Dal I	:	547	1988	2030	2333	2582	2670	
Dam II	:	2026						
		334	3093					

Table 5 (Con't)

Blo I	2267	2924	3030	3059			
Bly I	1369	1700	1806	1919	1922	2066	3255
Bly I*	1070	1276	1279	2026	2050	2683	
Bbv II	1875	2730					
Dg I	2106	2540					
Din I	17	342	956	1054	1140	3101	
Din I*	329	955	1052	2366	3000		
Dsp II	900	970	2979				
Dsp HI	2414						
Dsp HII	354						
Dst HR	215	528	638	806	1539	1552	1673
Cau II	6	339	340	736	769	1321	1986
Cfr 10I	374	2105	2519	2699	3050	3067	3308
Cfr I	2024	2529	2937	3069	3173		
Cla I	3446						
Cvi JI	192	265	272	343	350	361	386
Cvi QI	1393	1398	1534	1632	1658	1676	1687
Dde I	2026	2063	2145	2189	2210	2215	2353
Dpn I	2488	2518	2531	2552	2597	2641	2785
Dra II	2947	2985	2999	3071	3140	3175	3298
Dra III	211	3306					
Dsa I	135	571	661	717	1015	1424	1888
Eco 31I	11	238	336	950	962	1040	1048
Kco 47III	1026	2695	2976	3230			
	2342	2373	2645	3004	3095	3122	
	1988	2030	2945				
	295	331					
	345	2021	2940				
	615						
	1026	2695	2976	3230			
					1134	2010	232

Table 5 (con't)

Table 5 (Con't)

Table 5 (Cont'd)

Hae I	:	101	180	223	300	406	617	994	3414	3436
Hat I	:	2016	2114	3210						
Lae I	:	2187	2541	2701	3069					
Nar I	:	2264	2921	3035	3056					
Nco I	:	345								
Nle I	:	3239								
Nla III	:	168	232	349	382	565	620	912	982	1702
										1681
										201
Nla IV	:	2112	336	343	549	1631	1670	1989	2032	2146
										2181
										221
Nru I	:	2498								
Nsp BII	:	412	1115	1360	2331					
Nsp BI	:	382	1702	2910						
Pf1 HI	:	295	2105	2154						
Ple I	:	376	1807							
Ple I*	:	1322	2831							
Pma CI	:	331								
PPU HI	:	1998	2030							
Pss I	:	1991	2033	2949						
Rsa I	:	212	3307							
Sal I	:	370	2817							
Sce PI	:	6	215	339	340	528	638	736	769	806
										1321
										153
1552	1673	1986	2028	2212	2411	2936	3300	3340		

37bis

Table 5 (Cont'd)									
Sdu I	:	141	345	1300	2007	2290	2005	2907	3001
Sca I	:	5	330	345	1530	2021	2099	2301	3196
Sfa WI	:	650	818	2445	2020	3231	3344	2940	3339
Sfa WI*	:	420	1601	2038	2433	3054	3066	3255	335
Bma I	:	340							
Bph I	:	382	2910						
Bso II	:	4	213	337	338	526	636	734	767
Stu I	:	361							
Sty I	:	345	2099						
Taq I	:	254	371	666	1600	2202	2343	2934	3298
Taq IIB	:	1802							
Taq IIB*	:	2804							
Tth1111I	:	40	1107						
Tth1111I*	:	686	1075	1114					
Xba I	:	364							
Xho II	:	9	334	948	960	1046	1057	3093	
Xma I	:		338						
Xma III	:	2529							
Xmn I	:	467							

Total number of cuts is : 743.

Table 5 (con't)

List of non cutting selected enzymes.

<i>Xba</i> II	,	<i>Apa</i> II	,	<i>Avr</i> II	,	<i>Bbv</i> II*	,	<i>Bcl</i> I	,	<i>Bgl</i> I	,	<i>Bsp</i> MI*
<i>Bss</i> III	,	<i>Bst</i> BII	,	<i>Bst</i> XI	,	<i>Eco</i> 3II*	,	<i>Esp</i> I	,	<i>Hpa</i> I	,	<i>Hlu</i> I
<i>Hae</i> I	,	<i>Nde</i> I	,	<i>Not</i> I	,	<i>Nsi</i> I	,	<i>Pst</i> I	,	<i>Pvu</i> I	,	<i>Pvu</i> II
<i>Rsr</i> II	,	<i>Sac</i> I	,	<i>Sac</i> II	,	<i>Sau</i> I	,	<i>Sca</i> I	,	<i>Sci</i> I	,	<i>Sfi</i> I
<i>Sna</i> BI	,	<i>Spe</i> I	,	<i>Sph</i> I	,	<i>Ssp</i> I	,	<i>Taq</i> IIA	,	<i>Taq</i> IIA*	,	<i>Tth</i> IIII
<i>Vsp</i> I	,	<i>Xba</i> I	,	<i>Xho</i> I								

Total number of selected enzymes which do not cut: 38

EXAMPLE I: Purification of the TMA complex of M. paratuberculosis (A36), characterization of the proteic part of A36, identification of the 34 kDa protein and development of A36 based immunoassay:

MATERIALS AND METHODS

Bacteria:

The following mycobacteria were used: M. paratuberculosis strain 2E and 316F (from Dr. F. Saregaard, National Veterinary Institute, Oslo, Norway; Saregaard F. et al., 1985, "Control of paratuberculosis (Johne's disease) in goats by vaccination" 116:439-441); M. avium serotype 4 (from Dr. F. Portaels, Institute of Tropical Medicine, Antwerpen, Belgium) (Shaefer W.B., 1965, "Serologic identification and classification of the atypical mycobacteria by their agglutination", Am. Rev. Resp. Dis. suppl. 92:85-93); M. bovis strain BCG GL2 (from Dr. Weckx, Pasteur Institute, Brussels, Belgium) and M. phlei strain AM76 (from Dr. M. Desmecht, National Institute for Veterinary Research, Brussels, Belgium). The 168 strain of B. subtilis was used as control ATCC n° 33234.

Preparation of bacterial cytoplasms:

Bacterial suspensions in buffered saline (100 mg wet weight cells/ml 0.15 M NaCl 0.02 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.5 containing 10 mM phenylmethylsulfonyl fluoride) were disrupted by sonication (15 min treatment with a 500-W ultrasonic processor, Vibra cell from Sonics and Materials Inc, Danbury, Co USA (3 min sonication for B. subtilis)). Homogenates were centrifuged (5000 x g, 10 min, 4°C), and supernatants (i.e. mycobacterial cytoplasms) were stored at -20°C and used as sources of antigens.

Purification of TMA complexes:

The supernatant (about 4.5 mg proteins/ml) was submitted to RNAase digestion (10 µg enzyme/100 µg wet

weight bacteria, 30 min, 37°C) and fractionated by gel exclusion chromatography on Sepharose 6B columns (Pharmacia, Uppsala, Sweden) equilibrated with buffered saline, as previously detailed (Cocito C. et al., 1986, "Preparation and properties of antigen 60 from Mycobacterium bovis BCG" Clin. Exp. Immunol. 66:262-272). TMA complexes (thermostable macromolecular antigen complexes) were found within the excluded fractions (which contained on the average 0.5 mg soluble proteins/ml). Solutions of TMA (with 1 mM phenylmethylsulfonyl fluoride as conservative) were stored at -20°C.

Purity of TMA complexes was checked by crossed immunoelectrophoresis, according to the reference systems (Closs O. et al., 1980, "The antigens of Mycobacterium bovis, strain BCG, studied by crossed immunoelectrophoresis: a reference system" Scand. J. Immunol. 12:249-263 ; Gunnarsson E. et al., 1979, "Analysis of antigens in Mycobacterium paratuberculosis" Acta Vet. Scand. 20:200-215).

For this purpose agarose gels (1% type 2 agarose from Sigma, St Louis, Mo) on glass plates (5 by 7 cm) were used, the top gel containing 200 µl of rabbit anti-mycobacterial homogenate. Mycobacterial antigen (10 µl of samples containing 0.5 mg TMA/ml) was applied to a corner well and electrophoretic runs were made as described (1 h, 8 V/cm, 15°C in 1st dimension; 3 V/cm, 18 h, 15°C in 2nd dimension). Slants were washed, dried, stained with Coomassie blue and photographed.

#### Animal sera:

For production of polyclonal antisera, mycobacterial homogenate or TMA preparations (10 µg soluble proteins/0.5 ml buffered saline emulsified with equal volume of incomplete Freund adjuvant) were repeatedly injected (6 inoculations at 1-week intervals) into rabbits by subcutaneous way.

The antibody titer of the sera was evaluated by an immunoenzymometric procedure (see below).

Here is thus obtained a polyclonal anti-TMA complex antiserum, more particularly anti-A36 antiserum, and a polyclonal anti-homogenate antiserum referred to in the Western blotting test.

Four kinds of sera from bovines either healthy or at different stages of the Johne's disease were used: a) healthy controls with no sign of mycobacterial infection and with negative tests of coproculture and complement fixation; b) asymptomatic non-excretory stage I of the disease (a case which appeared negative at the moment of sampling but became positive later); c) asymptomatic excretory stage II of the disease (positive coproculture with no clinical signs of disease); and d) symptomatic excretory stage III of the disease (with positive complement fixation test). These sera were provided by the National Institute of Veterinary Research (Dr. M. Desmecht, Brussels, Belgium) and the Center of Veterinary Medicine (Dr. B. Limbourg, Erpent, Belgium).

**Electrophoretic fractionation and Western blotting of TMA proteins:**

The protein moiety of TMA complexes was fractionated by electrophoresis on 10% polyacrylamide gels, in the presence of Na dodecyl sulfate (SDS-PAGE procedure) (Laemmli U.K., 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4" Nature 227:680-695). Protein samples (25 µg soluble polypeptides in 50 µl 0.125 mM Tris-HCl pH 6.8 containing 5% w/v SDS, 20% v/v glycerol, 10% V:V β-mercaptoethanol and 0.05% bromophenol blue) were boiled for 5 min and then applied to vertical gel slabs. Molecular weight protein markers (Sigma Chem. Co., St Louis, Mo) were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate

dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.2 kDa). Electrophoretic runs (4 h, 50 V, 20°C) were made in a vertical unit (LKB, Bromma, Sweden). Protein bands were visualized by staining with Coomassie brilliant blue. Controls of total cytoplasmic proteins were run in parallel with TMA samples.

Electrophoresed proteins were transferred from polyacrylamide gels to nitrocellulose membranes (BA 85, Macherey-Nagel, Germany) by the use of a transblot-unit (217 multiphor 2, LKB, Bramma, Sweden).

Transfer buffer contained 20% methanol, 0.039 M glycine and 0.048 M Tris base pH 8.8, and runs were made at 10 V for 2 h. Transblotted proteins were identified by reaction with a primary antibody (either polyclonal rabbit antiserum [1/1500] or bovine serum [1/100]) and then with a labeled secondary antibody.

Transblotted nitrocellulose sheets were first incubated for 30 min with TBS buffer (0.5 M NaCl, 0.023 M Tris-HCl pH 7.5) containing 3% w/v gelatin and then for 3 h with the primary antibodies diluted with TBST buffer (TBS containing 0.05% v/v Tween 20) and 1% w/v gelatin. After repeated washings with TBST, sheets were incubated for 2 h with secondary IgG (1/400 diluted preparations of peroxidase-labeled anti-rabbit, or anti-mouse or anti-cow IgG, Dako, Copenhagen, Denmark), followed by washings with TBST and TBS buffers. A color reaction was developed by addition of  $\alpha$ -chloronaphthol (Bio-Rad Laboratories, Richmond, Cal) in the presence of hydrogen peroxide. The color reaction was stopped by washing sheets with distilled water. A similar protocol was used for antigens directly spotted on nitrocellulose membranes (dot-blot analysis). Reference samples of transblotted total proteins and molecular weight markers were visualized by India ink staining (10% solution of fount India, Pelikan, Germany, in 0.2

M NaCl, 0.05 M Tris-HCl pH 7.4 containing 0.3% v/v Tween 20) for 30 min (Hancock K. et al., 1983, "India ink staining of proteins on nitrocellulose paper" Anal. Biochem. 133:157-162).

Immunoassay for determination of anti-mycobacterial Ig:

Multiwell microtiter plates (Microwell Module, Nunc, Denmark) were coated either with purified A36 or with M. paratuberculosis total cytoplasm (i.e. supernatant) (0.5 µg soluble proteins/50 µl 0.05 M Na carbonate buffer pH 9.6/well). Air dry wells were saturated with bovine serum albumin (0.1% w/v BSA in 0.15 M NaCl, 1 h, 37°C). Increasing dilutions of serum to be tested in 0.15 M NaCl 0.02 M Na phosphate buffer pH 7.2 0.005% Tween 80 (PBST buffer) were added (50 µl/well, 1 h, 37°C), optimal dilutions being identified by checker board titration. Horse-radish peroxidase-labeled swine anti-rabbit, or rabbit anti-cow antiserum (Dako, Copenhagen, Denmark) were added (50 µl of 1/400 IgG dilution in PBST/well, 1 h, 37°C). Excess reagent was removed by 5 buffer washings. After incubation with the peroxidase reagent (50 µl per well of a 17 mM Na citrate buffer pH 6.3 containing 0.2% O-phenylenediamine and 0.015% H<sub>2</sub>O<sub>2</sub>, 30 min, 37°C in the dark), the reaction was stopped (50 µl 2 M H<sub>2</sub>SO<sub>4</sub>) and samples were spectrometrically measured (Plate reader SLT 210 from Kontron Analytical, U.K.). Results were recorded as ELISA absorbance values ( $A_{450\text{nm}}$ ).

In some experiments, cross-reactive Ig were removed by incubation (18 h, 4°C) with either purified TMA preparations (0.2 mg protein/ml of serum) or bacterial homogenates or intact mycobacteria (equivalents of 2 mg dry weight bacteria/ml of serum). Absorbed preparations were checked by dot-blot trials before application in immunoblot or immunoassay.

Immune electron microscopy:

Suspensions of mycobacteria in water ( $5 \times 10^7$  cells/5  $\mu\text{l}$ ) were placed on carbon-formvar 200-mesh copper grids and air dried. Grids were serially incubated with: a) bovine serum albumin (3% solution in buffered saline, 30 min, 37°C); b) anti-TMA complex rabbit antiserum (a 10<sup>3</sup> dilution of Ig in buffered saline with 0.05% Tween 20, 2 h, 37°C); c) sheep anti-rabbit biotinylated Ig (1/200 dilution of Ig from Amersham, U.K., in buffered saline-Tween, 1 h, 20°C); d) gold-labeled streptavidin (a 1/20 dilution of a preparation from Amersham, U.K.) (Cloeckaert A. et al., 1990, "Identification of seven surface-exposed Brucella outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay" Infect. Immun. 58:000-000). Grids were analyzed in a transmission electron microscope (Philips CM 10).

RESULTSPurification of TMA complexes and preparation of anti-TMA antisera:

The TMA complex of M. paratuberculosis (A36) has been prepared from the total homogenate. Cytoplasm fractionation by gel exclusion chromatography yielded said TMA complex within the exclusion fraction. The immunoelectrophoretic patterns of total cytoplasmic antigens (supernatant) (Figure 1(1)) and of the exclusion fraction (Figure 1(2)) are compared. From these tracings, which were obtained with polyclonal antisera elicited by inoculation of rabbits with whole mycobacterial homogenate, the purity of the A36 preparation can be inferred.

A similar protocol was used for preparation of other antigens of the TMA group from M. avium, M. bovis and M. phlei, which were used for comparative analysis.

The polyclonal antisera corresponding to the TMA complexes have also been prepared. The purity of these Ig preparations was checked by crossed immunoelectrophoresis: using total cell homogenates as antigens in every case, a single immunoprecipitinogen line corresponding to the TMA complex was obtained (patterns not shown, mimicking that of Figure 1(2)). It is to be noted that subcutaneous injection of TMA complex preparations invariably induced the synthesis of high titer antisera (ELISA absorbance higher than 2.5 for dilutions at  $10^{-5}$ ), a result which stressed the high immunogenicity of these antigen complexes.

**Development of A36-based serological assay for paratuberculosis:**

The availability of A36 has prompted the development of an enzymometric ELISA-type immunoassay for paratuberculosis. Accordingly, multiwell plates were coated with A36 and incubated with sera of infected animals. Peroxidase-labeled rabbit anti-bovine IgG were added as second antibody, and the color developed after addition of peroxidase reagent was measured spectrophotometrically, as detailed in Materials and Methods. A comparative survey was made in parallel with A36 and with total cytoplasm (supernatant) of M. paratuberculosis (equal amounts of proteins were used for the two assays).

All the sera of infected animals (stages II and III of the Johne's disease) yielded a positive answer (values of 0.84 to 2.25 units) to both types of the ELISA assay (Figure 3). On the contrary, uninfected animals were invariably negative (values lower than 0.38 units). With A36-ELISA, considerably higher absorbance values (1.44 to 2.25 units) were obtained than with the total cytoplasm-ELISA (0.84 to 1.65).

These results suggest the immunodominance of the A36 antigen in the Johne's disease, and the usefulness of the A36-based ELISA as a diagnostic assay.

**Peripheral location of the TMA complex in mycobacteria:**

The observed immunodominance of A36 is more compatible with a surface component than with an antigen complex located in the cytoplasm. However, a transfer of TMA complex through the envelope and its protrusion at the cell surface is conceivable.

The use of the immunolectron microscopy methodology has allowed a direct approach to this problem. Multiplying cells of M. paratuberculosis were incubated with anti-A36 Ig from immunized rabbits. Cell-bound primary antibodies were revealed by secondary swine anti-rabbit IgG labeled with colloidal gold. Electron micrographs show the presence of antigen reactive spots on the surface of mycobacteria (results not shown).

These data indicate that part of the TMA complex does indeed occur within the cell wall and is presented on the cell surface.

**Immunological crossreactivity of A36 and other TMA antigens:**

In the preceding section, the development of a A36-based ELISA assay for titration of anti-mycobacterial antibodies has been described. The possible use of this assay in Veterinary Medicine relies on its specificity with respect to: a) other mycobacteria which are usual hosts of the intestinal tracts of ruminants; and b) M. bovis, and M. tuberculosis which can cause tuberculosis in cattle (compulsory slaughtering of PPD-positive cattle). This problem was approached by evaluating the crossreactivity of TMA complexes from different mycobacteria with two procedures (see Table 1).

A first series of assays was carried out with microtitration plates coated with the TMA complex from M. avium, M. bovis, M. paratuberculosis and M. phlei. All these plates were used to titrate a single anti-A36 antiserum, a procedure yielding an evaluation of the percentage of shared TMA epitopes. Considering the autologous reaction (A36-anti A36 IgG) equal to 100, percentage of homology of M. paratuberculosis TMA complex with the TMA complex of M. avium and bovis was very high; it was much lower for M. phlei TMA complex.

When the A36-based ELISA assay was repeated with anti-A36 antiserum previously absorbed by different mycobacterial TMA complexes, an evaluation of the A36 specific epitopes was obtained. From Table 1, it results that the percentage of specific epitopes was low when the A36 was compared to the TMA of M. avium and M. bovis, it was high when compared to the TMA of M. phlei.

**TABLE 1 : CROSSREACTING AND SPECIES SPECIFIC EPITOPES IN THE TMA COMPLEXES OF FOUR MYCOBACTERIA**

**TMA in ELISA**

Parameter	Coating reagent (plate)*	Absorbing reagent (antiseraum)* <sup>b</sup>	ELISA units (A <sub>450nm</sub> ) <sup>c</sup>	Cross-reacting epitopes (%) <sup>c,d</sup>
<b>A. Crossreactivity</b>	<u>M. parat.</u>	-	2.367	100
	<u>M. avium</u>	-	2.376 ( $\pm 0.247$ )	100 ( $\pm 13$ )
	<u>M. bovis</u>	-	2.240 ( $\pm 0.181$ )	96 ( $\pm 10$ )
	<u>M. phlei</u>	-	1.083 ( $\pm 0.156$ )	49 ( $\pm 8$ )
<b>B. Specificity</b>	<u>M. parat.</u>	<u>M. parat.</u>	0.462	0
	<u>M. parat.</u>	<u>M. avium</u>	0.574 ( $\pm 0.197$ )	7 ( $\pm 11$ )
	<u>M. parat.</u>	<u>M. bovis</u>	0.603 ( $\pm 0.238$ )	10 ( $\pm 13$ )
	<u>M. parat.</u>	<u>M. phlei</u>	1.073 ( $\pm 0.141$ )	48 ( $\pm 8$ )

\* TMA preparations from different mycobacteria (0.5 µg/well) were used to coat microfiltration plates

<sup>b</sup> anti-AJ6 antiseraum was pre-absorbed (samples B) or not (samples A) with TMA complex from different mycobacteria

<sup>c</sup> to plates coated with AJ6 (samples B) or with different TMAs (samples A) anti-AJ6 antiseraum (1/15000 dilution) was added, and bound Ig were revealed by a second labeled antibody

<sup>d</sup> percentage of crossreacting or specific epitopes calculated on a logarithmic scale.

These results show the lack of species-specificity of the A36-ELISA as a diagnostic reagent for the Johne's disease. They suggest, however, the possible occurrence of A36 components endowed with such a specificity.

**Immunodominance and specificity of the A36 proteins:**

The species specificity, which was missing at the level of the complete A36 antigen complex, was sought with respect to its protein components. The TMA complexes from M. avium, M. bovis, M. paratuberculosis and M. phlei were isolated, and their protein components were fractionated by polyacrylamide electrophoresis. A similarity of M. avium and M. paratuberculosis tracks is apparent, whereas those of M. bovis and M. phlei TMA were clearly different to the M. paratuberculosis track.

When fractionated A36 proteins were immunoblotted with anti-A36 antiserum, a dozen of major polypeptides were stained, most of them located in the 28-42 kDa region. Immunoblotting with anti-A36 antiserum pre-absorbed with a lysate of M. phlei yielded 5 polypeptide bands; they were 3 in the case of M. bovis and one with M. avium. Table 2 provides a comparative evaluation of the main A36 components according to two properties: immunogenicity level (staining intensity by pooled sera of infected bovines) and species specificity (lack of cross-reactivity with the other mycobacteria). Eleven major components of 22 to 74 kDa are listed: two of them (of 23 and 31 kDa) containing specific epitopes with respect to the tested organisms except M. avium, and one of 34 kDa containing specific epitopes with respect to all of the tested organisms including M. avium.

TABLE 2 : IMMUNOLOGICAL CHARACTERISTICS OF SOME PROTEINS OF THE TMA COMPLEX OF  
MYCOBACTERIUM PARATUBERCULOSIS (A36)

Protein <sup>a</sup> (kDa)	Immunogenicity <sup>b,c</sup> (levels in hosts)			specificity <sup>d</sup> towards		
	anti-A36	rabbit	infected bovines	<u>M. avium</u>	<u>M. bovis</u>	<u>M. phlei</u>
	I	II	III			
74	++	-	-	no	no	no
52	+	-	-	no	no	no
41	+	+	+	no	no	yes
40	+++	+	+	no	no	no
37	++	++	-	no	no	yes
35	+	++	++	no	no	yes
34	++	++	++	yes	yes	yes
31	++	++	++	no	yes	yes
29	++	++	-	no	no	yes
23	++	-	+	no	yes	yes
22	+	-	++	no	no	yes

• A36 was dissociated and protein components were fractionated by SDS-PAGE electrophoresis and identified by immunoblotting

° degree of immunogenicity for rabbits and cows was evaluated from the intensity of immunoblot staining with the corresponding sera

c sera from cattle affected by different stages of the Johnne's disease: I, asymptomatic-excretory; II, asymptomatic-excretory; and III, symptomatic-excretory forms

d crossreactivity was expressed by a no, and specificity by a yes.

The immunological relevance of the latter protein was checked by immunoblot analysis of A36 proteins with infected bovine sera: a major band at the level of the 34 kDa marker was observed (Figure 4, lanes 4, 5, 6 and 7). This band was missing in the control (lane 8 with healthy bovine serum).

It is thus evident that the 34 kDa protein component of the TMA complex is immunodominant in cattle, relevant to Johne's disease, and containing species-specific epitopes with respect to related mycobacteria.

The present invention enables to develop a A36 based ELISA test for paratuberculosis: its ability to reveal the presence of a mycobacterial infection in cattle has been proven in Figure 3. Basic requirements for the use of a given antigen as reagent for immunoassays of medical interest are: 1) its immunodominance; 2) its relevance to the targeted disease; and 3) its specificity. Requirements 1 and 2 were therefore fulfilled by the A36 based-ELISA. Requirements 1 to 3 are completely fulfilled by the p362 polypeptide which is part of the 34 kDa protein belonging to A36, as described hereafter.

EXAMPLE II: Isolation of clone a362 expressing a 10 kDa polypeptide (p362), DNA sequencing of the insert of clone a362 and testing of p362 in an ELISA for Johne's disease:

MATERIAL AND METHODS

Cloning vectors

The following types were used: λgt11 (Young R.A. and Davis R.W., 1983, "Yeast RNA polymerase II genes: isolation with antibody probes" Proc. Natl. Acad. Sci. USA 80:1195-1198) and pUEX2 (Brennan G.M. et al., 1987, "pUEX, a bacterial expression vector related to pEX with universal host specificity" Nucl. Acids Res.

15:10056) and pmTNF-MPH (see Figures 9a, 9b and Table 5) as expression vectors, and the Blue-Script SK<sup>+</sup> as sequencing vector (Stratagene).

#### Bacteria

Mycobacterium paratuberculosis 19698 (from the American Type Culture Collection). M. paratuberculosis: strain 2887 (Crohn): ATCC n° 43015. M. avium serotype 4, M. avium serotype 2, M. avium serotype 8 (Schaefer W.B., 1965, "Serologic identification and classification of the atypical mycobacteria by their agglutination" Am. Rev. Resp. Dis. suppl. 92:85-93). M. tuberculosis H37rv: ATCC n° 25618. M. gordonaiae: ATCC n° 14470. Brucella abortus B3 (Cloeckaert A. et al., 1990, Infect. Immun. 58:3980-3987). Strains of Escherichia coli: Y1089 ( $\Delta$ (lacU169),  $\Delta$ (lon), hflA150 (chr::Tn10), (pMC9), (rK<sup>-</sup>, mK<sup>-</sup>)), Y1090 ( $\Delta$ (lacU169),  $\Delta$ (lon), sup F, (trpC22::Tn10), (pMC9), (rK<sup>-</sup>, mK<sup>-</sup>)), MC1061 ( $\Delta$ (lacX74), galU, galK, (rK<sup>-</sup>, mK<sup>-</sup>)) and DH5αF' (F', (rK<sup>-</sup>, mK<sup>-</sup>), supE44, lacZAM15,  $\Delta$ (lacZYA argF) U169), K12AH, ATCC 33767 (lacZ(am)  $\Delta$ (bio uvr B) (λ Nam7 am53 cI 857 ΔH1) rpsL20).

#### Antisera

Rabbit anti-M. paratuberculosis antiserum was from Dako (Copenhagen, Denmark, lot n° 014). Sera from paratuberculosis-infected cattle were provided by Dr. M. Desmecht (National Institute for Veterinary Research, Brussels) and Dr. B. Limbourg (Erpent, Center of Veterinary Medicine, Belgium).

Polyclonal antisera against whole homogenate of M. avium serotype 4, M. bovis BCG, and M. phlei, as well as those against the TMA complex and βgal-p362 (recombinant polypeptide of the invention fused to β-galactosidase hereafter described) were produced by repeated subcutaneous inoculations into rabbits (10 µg proteins/0.5 ml buffered saline emulsified with equal

volume of incomplete Freund's adjuvant, 6 inoculations at 1-week intervals).

Purification of *M. paratuberculosis* DNA:

Suspensions of bacteria (10 mg in 0.5 ml of 100 mM NaCl, 1 mM EDTA, 50 μM Tris-HCl pH 7.4) were incubated sequentially with lysozyme (25 μl of 20 mg/ml, 14 h, 50°C), pronase (25 μl of 20 mg/ml, 1 h, 37°C), and SDS (25 μl of 20%, 1 h 37°C). Mixtures were extracted with chloroform-isoamyl alcohol (24:1, vol:vol), water-saturated phenol, and ether. After incubation with ribonuclease (5 μl of 2 mg/ml, 1 h, 37°C), DNA was purified on columns of Sephadex G50 (equilibrated with 4,8 mM sodium phosphate pH 6,8) and hydroxyapatite (washed with 8 M urea, 0,1 M sodium phosphate buffer pH 6,8 containing 1% SDS, and then with 4,8 mM sodium phosphate pH 6,8, and eluted with 480 mM sodium phosphate pH 6,8).

Construction of a λgt11 library of *M. paratuberculosis*:

*M. paratuberculosis* DNA was sheared to average length segments of 0,5 to 1,5 kb (Vibra Cell ultrasonicator 60 W, 2 sec). Shearing was monitored by agarose gel electrophoresis. EcoR1 sites were methylated with EcoR1 methylase (5 μg of sheared DNA in 50 μl of buffer (50 mM Tris-HCl pH 7,5, 1 mM Na<sub>2</sub>EDTA, 5 mM dithiothreitol, 50 μM S-adenosyl-L-methionine and 10 units of EcoR1 methylase). Methylation was pursued for 30 min at 37°C, and stopped by 10 min incubation at 70°C. Blunt-end DNA fragments were obtained by incubation with T4 DNA polymerase (5 μl of 0,1 M MgCl<sub>2</sub>, 2,5 μl of 1 mM dTNPs, 1 μl of 1 M(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 20 units of T4 DNA polymerase per 40 μl methylation reaction medium; 20 min incubation at 37°C). EDTA (15 mM final concentration) was added, reaction mixture was extracted with phenol/chloroform twice, and the aqueous phase was extracted with ether. After addition of sodium acetate 0,3 M final concentration, DNA was

precipitated with 2 vol of EtOH at -20°C and washed with 70% EtOH. DNA pellet was dissolved in buffer (10 µl of 100 mM Tris-HCl pH 7,5, 20 mM MgCl<sub>2</sub>, 20 mM dithiothreitol), phosphorylated EcoR1 linkers (200 µg/ml) were added, followed by addition of PEG 6000 (final concentration 15%), 1 mM ATP (final concentration) and 2 units of T4 DNA ligase, and the reaction mixture was incubated overnight at 12°C. This mixture was incubated at 37°C with an excess of EcoR1, and DNA fragments were purified from linker excess on Sephadex G25. The DNA solution thus obtained was extracted sequentially with phenol/chloroform and ether, precipitated, and washed with ethanol. DNA pellet (0,5 µg) was dissolved in TE buffer (10 mM Tris-HCl pH 7,5, 0,1 mM EDTA) and ligated (18 h, 4°C) with 1 µg of dephosphorylated EcoR1-digested λgt11 DNA (Promega). Methylation, ligation, and digestion steps were controlled by agarose gel electrophoresis. Phage packaging of cloned DNA was obtained with the Stratagene gigapack extract.

Screening of the λgt11 library and dot-blot technique:

After infection of E. coli Y1090 by the recombinant phage mixture and spreading them out over the plate, they were incubated for 3-4 h at 42°C.

For identification of recombinant phages, IPTG (isopropylthio β-galactopyranoside) (10 mM) saturated nitrocellulose filters were placed directly on the surface of the overlay plates containing the plaques and incubated for 18 h at 37°C (Young R.A. and Davis R.W., 1983, "Yeast RNA polymerase II genes: isolation with antibody probes" Proc. Natl. Acad. Sci. USA 80:1195-1198). After spotting of control antigens (1 µg) and washing for 10 min with TBS buffer (0,5 M NaCl, 0,023 M Tris-HCl pH 7,5), filters were incubated for 30 min with the same buffer containing 3% (w/v) gelatin and then with the rabbit anti-M. paratuberculosis

antiserum (Dako) previously diluted with TBST buffer (TBS buffer containing 0,05% (v/v) Tween 20) containing 1% (w/v) gelatin. After washing, filters were incubated for 1 h with 1/400 dilutions of peroxidase-labeled anti-rabbit Ig. After repeated washing with TBST and TBS, the peroxidase substrate  $\alpha$ -chloronaphthol (Bio Rad Laboratories, Richmond, Calif.) and hydrogen peroxide were added. Reaction was stopped by washing with distilled water. Plaques corresponding to reactive spots on the filters were picked off, transferred to SM medium (100 mM NaCl, 10 mM MgSO<sub>4</sub>, 20 mM Tris-HCl pH 7,4) and purified by repeated passages in E. coli Y1090. Recombinant clones were then further characterized with respect to their antigenicity (incubation with bovine sera and anti-A36) and their specificity (incubation with antibodies directed against homogenate of M. avium, M. bovis and M. phlei) using the same procedure as described above.

A similar technique was used for dot-blot experiments in which the specificity of the recombinant polypeptide p362 was tested with respect to different mycobacteria: spots of mycobacterial homogenates on nitrocellulose membranes were incubated with anti- $\beta$ gal-p362 Ig.

High level expression of fusion protein in E. coli:

Colonies of E. coli Y1089 lysogenized with the appropriate  $\lambda$ gt11 recombinants were multiplied at 30°C in Luria-Bertani medium ( $A_{600\text{nm}}=0,5$ ). After heat shock (20 min at 45°C), production of  $\beta$ -galactosidase fusion proteins of the invention was induced by the addition of 10 mM IPTG (final concentration) and further incubation (60 min at 37°C). Cells harvested by centrifugation were suspended in buffer (10 mM Tris-HCl, pH 8,2, 2 mM EDTA) and rapidly frozen in liquid nitrogen.

For enhanced expression,  $\lambda$ gt11 inserts were subcloned into the expression vector pUEX2 (Brennan G.M. et al., 1987, "pUEX, a bacterial expression vector related to pEX with universal host specificity" Nucl. Acids Res. 15:10056), commercially available from Amersham, which was used to transform E. coli MC1061 (Maniatis, Molecular Cloning). Single colonies of transformed E. coli were grown at 30°C to  $A_{600}=0,3$  and heat-shocked (90 min at 42°C). Harvested cells were lysed by sonication and frozen in liquid nitrogen.

Protein fractionation and immunoblotting:

The TMA complex and recombinant proteins were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (SDS PAGE) (Laemmli, U.K. 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", Nature 227:680-695).

Fractionation on 7,5 or 10% acrylamide gels was carried out in a 2001 vertical electrophoresis unit (LKB-Produkter AB, Bromma, Sweden) (4 h, 50 V, 20°C). Molecular weight protein markers (Sigma, St Louis, Mo) were: myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase B (97,4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and  $\alpha$ -lactalbumin (14.2 kDa). Protein bands were stained with Coomassie brilliant blue. Electrophoresed proteins were transblotted (LKB 217 Multiphor 2 Electrophoresis System, 10 V, 2 h, with buffer 20% methanol, 0,039 M glycine and 0,048 M Tris base, pH 8.8) onto nitrocellulose membranes. Mycobacterial antigens were visualized by sequential incubation with polyclonal rabbit antisera (anti-A36 for recombinant mycobacterial antigens fused to  $\beta$ -galactosidase or anti- $\beta$ gal-p362 for TMA proteins) and peroxidyase-labeled anti-rabbit Ig (Dako, Copenhagen, Denmark) (1/400 dilution). Total

protein blotted on the membrane was visualized by staining with India ink.

DNA Sequencing:

Sequence analysis of the DNA insert of the recombinant clone a362 was done by the primer extension and dideoxy termination method (Sanger F. et al., 1977, "DNA sequencing with chain terminating inhibitors", Proc. Natl. Acad. Sci. USA 74:5463-5467), after subcloning of the  $\lambda$ gt11 insert into the sequencing vector pBluescript SK<sup>+</sup> (Stratagene). Sequencing reactions were performed with T7 DNA polymerase and different primers (universal, reverse, SK, and KS primers from Deaza Kit, Pharmacia, Uppsala, Sweden). Computer-aided analysis of nucleic acid and polypeptide sequences were performed with the program COD-FICK (PC-GENE, Intelligenetics, USA). Homology searches were performed on DNA level in EMBL bank (release 29) and UGEN bank (release 70-29) (Intelligenetics Inc., CA-USA), and on protein level in PIR bank (release 31) and Swiss Prot (release 20). No homologous sequences were found.

Serological analysis (ELISA) with recombinant polypeptides:

Multiwell microtiter plates (Microwell Module, High binding Capacity, Nunc, Denmark) were coated with total cytoplasm of E. coli-a362 and with total cytoplasm of E. coli as a control. Four  $\mu$ g of soluble proteins / 50  $\mu$ l 0,05 M Na carbonate buffer pH 9,6 were coated per well. Plates were air dried overnight and saturated (0,1% serum albumin in 0,15 M NaCl, 1 h at 37°C). Dilutions of bovine Ig in PBST (0,15 M NaCl, 0,02 M phosphate buffer pH 7,2, containing 0,005% Tween 80) were added to plate wells (50  $\mu$ l, 1 h at 37°C). Peroxidase-labelled rabbit anti-cow Ig (Dako) (50  $\mu$ l, 1/400 dilution in PBST/per well) were added (1 h at 37°C). Excess of reagent was removed by 5 PBST

washings. After incubation with peroxidase reagent (50 µl/well of 0.2% O-phenylenediamine with 0.015% hydrogen peroxide in 0.017 M Na citrate buffer pH 6.3, 30 min, 37°C in the dark), the reaction was stopped with 50 µl 2 M H<sub>2</sub>SO<sub>4</sub>, and A<sub>450nm</sub> was measured in a colorimetric plate reader (SLT 210, Kontron Analytical, UK). Results were recorded as ELISA absorbance values. In some experiments, cross reactive Ig were removed by incubation (18 h at 4°C) with bacterial homogenate. Absorbed preparations were checked by dot-blot trials before applications in immunoblots or immunoassays.

#### Immune electron microscopy:

Suspensions of mycobacteria in water ( $5 \times 10^7$  cells/5 µl) were placed on carbon-formvar 200-mesh copper grids and air-dried. Grids were serially incubated with: a) bovine serum albumin (3% solution in buffered saline, 30 min, 37°C); b) anti-βgal-p362 rabbit antiserum (a 10<sup>-3</sup> dilution of Ig in buffered saline with 0.05% Tween 20, 2 h, 37°C); c) sheep anti-rabbit biotinylated Ig (1/200 dilution of Ig from Amersham, U.K., in buffered saline-Tween, 1 h, 20°C); d) gold-labelled streptavidin (a 1/20 dilution of a preparation from Amersham, U.K.) (Cloeckaert A. et al., 1990, "Identification of seven surface-exposed Brucella outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay", Infec. Immun. 58:3980-3987).

Grids were analyzed in a transmission electron microscope (Philips CM 10).

#### RESULTS

##### 1. Preparation of a genomic library of *M. paratuberculosis* and isolation of recombinant clones:

A genomic library of *M. paratuberculosis* has been prepared by the use of the expression vector λgt11. For this purpose, purified mycobacterial DNA was sonicated

under controlled conditions yielding segments of  $10^3$  bp on the average (0.5 to  $2 \times 10^3$ ). These fragments were methylated by EcoRI DNA methylase (efficiency of methylation was controlled by incubation with EcoRI), incubated with T4 DNA polymerase to obtain blunt-end DNA, and provided with EcoRI linkers by incubation with T4 DNA ligase. After EcoRI digestion, DNA segments were purified free of linker excess and inserted into EcoRI-cleaved  $\lambda$ gt11 by incubation with T4 DNA ligase (a step checked by gel electrophoresis). After packaging and infection of E. coli Y1090,  $7.5 \times 10^5$  recombinant clones (75% of total clones) were obtained, one third of which was screened with rabbit anti-M. paratuberculosis antiserum (Dako). After repeated purifications, ten recombinant clones were selected: three of them expressed TMA complex proteins, and seven produced epitopes of proteins not present within the TMA complex.

2. Analysis of antigenicity and specificity of polypeptides produced by recombinant clones:

Since cloning of M. paratuberculosis genes was aimed at producing polypeptides to be used as diagnostic reagents, it appeared essential to test the reactivity of recombinant clones towards sera of cattle affected by the Johne's disease. As shown in Table 3, all the selected clones reacted with sera of animals bearing one of the clinical forms of the disease. The strongest reactions were afforded by clones a4 and a362. On the contrary, no reactivity was observed with sera from healthy bovines.

TABLE 3  
Characteristics of clones expressing an antigenic polypeptide of M. paratuberculosis

Clones*	Antigenicity**			Specificity with respect to***		
	1	2	3	<i>M. avium</i>	<i>M. bovis</i>	<i>M. phlei</i>
a1	(+)	+	+	no	no	yes
a2	+	+	+	yes	yes	yes
a3	+	+	++	no	yes	yes
a4	++	++	++	no	no	yes
a5	+	+	+	no	yes	yes
a6	+	+	++	no	no	yes
a7	(+)	+	+	no	no	no
---	-----	-----	-----	-----	-----	-----
a361	+	+	++	no	yes	yes
a362	++	++	++	yes	yes	yes
a363	(+)	+	+	no	no	yes

\* only clones a361 to a363 express polypeptides belonging to the TMA complex.

\*\* detected by sera from asymptomatic and non excretory bovine (1), asymptomatic and excretory bovine (2) and symptomatic and excretory bovine (3); quantified as low reaction "(+)", good reaction "+" and very good reaction "++".

\*\*\* cross reactivity was expressed by a "no", and specificity by a "yes".

Another requirement of paramount importance was the specificity with respect to mycobacteria belonging to the saprophytic and pathogenic flora of cattle. Recombinant clones were tested for reactivity with

antisera against homogenates of M. avium, M. bovis and M. phlei. It was previously shown that the overall DNA homology levels of these three mycobacteria with respect to M. paratuberculosis were respectively 94, 52, and 19 percent (Hurley S.S. et al., 1988, "DNA relatedness of M. paratuberculosis to other members of the family of mycobacteriaceae", Int. Journal Syst. Bact. 38:143-146). Data in Table 3 indicate that, although all clones but one were specific towards M. phlei, only five of them were specific for M. bovis and two for M. avium.

In conclusion, only two of the selected clones, a2 and a362 fulfilled both requirements for species-specificity and relevance to Johne's disease. Moreover, only the latter clone reacted with anti-A36 antiserum and corresponded, therefore, to a A36 protein, presumably the 34 kDa protein previously identified as a TMA complex component with species-specific epitopes. The remaining part of this example relates to the characterization and use of clone a362.

### 3. Size of clone a362 insert and its expressed polypeptide p362:

EcoRI cleavage of DNA of clone a362 yielded an insert of about 500 bp devoid of internal EcoRI restriction sites (not shown).

E. coli Y1089 was lysogenized by the recombinant phage, and the synthesis of a chimaeric protein fused with  $\beta$ -galactosidase was induced by IPTG: a fusion protein of about 125 kDa ( $\beta$ gal-p362) was produced (Figure 5). Since  $\beta$ -galactosidase (116 kDa) misses 2 kDa in  $\lambda$ gt11, the recombinant polypeptide coded for by the insert of clone a362 (p362) is expected to be about 11 kDa in size. Consequently, only a roughly 300 bp portion of the 500 bp insert coded for such an 11 kDa polypeptide. This was confirmed by sequencing and

determination of the orientation of the insert DNA as described further.

4. Production of p362 recombinant polypeptide and evidence of its belonging to a 34 kDa protein of A36:

Since the production of the  $\beta$ -gal p362 by E. coli Y1089 containing the  $\lambda$ gt11-recombinant phage was only 2% of total proteins, the corresponding insert was recloned in a more favorable expression vector. For this purpose, the  $\lambda$ gt11 insert of the a362 recombinant clone was freed by incubation with EcoR1, purified by electroelution from an agarose gel (75% recovery), and recloned into the EcoR1 site of the expression vector pUEX2 (Amersham). In this case, production of  $\beta$ gal-p362 fusion protein in the transformed MC1061 strain of E. coli ( $6 \times 10^5$  transformants/ $\mu$ g DNA) was about 25% of total proteins.

After running the SDS-PAGE of the lysate from the transformed strains, the recombinant fusion protein was eluted from the polyacrylamide gel and used to elicit antibodies in rabbits (anti- $\beta$ gal-p362).

The protein components of the TMA complex from M. paratuberculosis were fractionated by electrophoresis on polyacrylamide gels (SDS PAGE). After transfer to nitrocellulose sheets, TMA proteins were incubated with anti- $\beta$ gal-p362. As shown in Figure 6, a major band corresponding to the 34 kDa protein of the TMA complex was immunolabeled: this was the unique TMA protein containing species-specific epitopes as above reported. A second band of about 31 kDa was stained to minor extent: it was also present in the immunoblots of TMA proteins with sera of infected cattle.

5. Localization of the p362 polypeptide at the bacterial surface:

Since the A36 antigen complex was previously shown to be present at the cell surface, a peripheral location of the p362 recombinant polypeptide would

further confirm the belonging of p362 recombinant polypeptide to a protein of the A36 complex. Electron micrographs show indeed the presence of the p362 polypeptide within the cell wall and its release during the declining growth phase (results not shown).

6. Assessment of the species-specificity of the recombinant polypeptide p362:

From what is above-mentioned, it is shown that the 34 kDa protein component of the TMA complex of M. paratuberculosis contains epitopes devoid of crossreactivity towards M. bovis, M. avium and M. phlei. Although the recombinant p362 polypeptide, which apparently represents a portion of the 34 kDa protein, is likely to be endowed of species-specificity, a more stringent confirmation is needed for a polypeptide forecast as reagent for serological tests. Consequently, the specificity of p362 was tested against two series of M. paratuberculosis and M. avium isolates from cattle as well as against certain Gram-positive and Gram-negative bacteria being usual hosts of bovine gut (Table 4).

The dot-blot experiment was carried out by spotting on a nitrocellulose membrane 2 µg samples of different bacterial homogenates. Membranes were then incubated successively with rabbit anti- $\beta$ gal-p362 antiserum and, after washing, with peroxidase-labeled swine anti-rabbit IgG. Spots were revealed by the peroxidase reaction. All of eight M. paratuberculosis isolates were positive, whereas the closely related organisms of the MAIS group were negative. None of the other tested mycobacteria gave a positive reaction, neither did the Nocardia and Brucella species (see Table 4).

TABLE 4 : SPECIFICITY OF pJ62 TOWARDS OTHER [MYCO]BACTERIA

Bacterium lysates	Anti- $\beta$ gal-pJ62	Bacterium lysates	Anti1- $\beta$ gal-pJ62
<b>- <i>M. paratuberculosis</i> :</b>			
2E	+		
316F	++		
ATCC 19698	++		
ATCC 43015	++		
2890 (bovine) (1)	++		
2891 (bovine) (1)	++		
2895 (goat) (1)	++		
172 28/66 (bovine) (2)	++		
- <i>M. avium</i> D4 (5)	-		
- <i>M. avium</i> serotype 4	-		
- <i>M. avium</i> serotype 8	-		
- <i>M. avium</i> serotype 2	-		
- <i>M. scrofulaceum</i> (1)	-		
- <i>Salmonella typhimurium</i> (3)	-		
<b>- <i>M. intracellulare</i> (1)</b>			
MAIS AJ (4)			
MAIS A84 (4)			
MAIS 8715 (4)			
MAIS 87537 (4)			
<i>M. bovis</i> BCG GL2			
<i>M. tuberculosis</i> H37rv (6)			
<i>M. phlei</i> AM76 (1)			
<i>M. leprae</i> (1)			
<i>M. fortuitum</i> M62 (1)			
<i>M. smegmatis</i> (1)			
<i>M. gordoniæ</i> ATCC 14430			
<i>Nocardia asteroides</i> (1)			
<i>Brucella abortus</i> B3 (3)			

(+) positive immunological reaction

(-) absence of reaction

- (1) Portaels IMTA (Institut de Médecine Tropicale, Anvers Belgique)
- (2) from Kaeckenbeeck DBUL (Département de Bactériologie, Université de Liège, Belgique)
- (3) from LINET ICP (Institut of Cellular Pathology, Belgique)
- (4) from Defoe IPB (Institut Pasteur du Brabant, Belgique)
- (5) from Saxegaard NVIN (National Veterinary Institute, Norway).
- (6) ATCC 25618

7. Sequencing of the cloned insert coding for polypeptide p362:

To sequence the 500 bp DNA segment coding for the polypeptide p362, the insert of clone a362 was isolated by EcoRI cleavage from the chimaeric vector  $\lambda$ gt11 and recloned into the Bluescript vector SK<sup>+</sup>. After transformation of E. coli DH5 $\alpha$ F', clones carrying inserts coding for p362 were selected.

The sequence of the insert showed the occurrence of a 507 bp DNA segment flanked by two EcoRI extremities (Figure 7C). The G+C content of this segment was 70%, in agreement with the 64% G+C of the whole M. paratuberculosis genome. The sequence in Figure 7C yielded two open reading frames in phase with the EcoRI sites: a 306 bp region (1 to 306) in one direction, and a 185 bp region (507 to 322) into opposite orientation. The program COD-FICK (PC-GENE) which takes in account the codon usage, confirmed the coding ability of the two open reading frames. They coded respectively for 10 kDa and 7 kDa polypeptides. The insert was subcloned in an expression vector in E. coli in both orientations. Only one orientation yielded an expression product reacting with the rabbit anti- $\beta$ gal-p362 antiserum. Restriction analysis led to the selection of the 306 bp open reading frame as being the one coding for the p362 polypeptide [10 kDa]. The selected coding region and the aminoacid sequence of polypeptide p362, corresponding to the carboxyterminal extremity of the 34 kDa protein are displayed in Figure 8.

8. Testing of p362 in an ELISA for Johne's disease:

The 10 kDa polypeptide (p362), endowed with species-specificity, and being part of the 34 kDa protein of A36, can be used as a specific test for paratuberculosis.

A preliminary test has been done using plates coated with total cytoplasm of E. coli-a362 containing p362. Bovine sera were preabsorbed to E. coli-control homogenate. Figure 2 shows that all sera from infected bovines react significantly with p362. On the contrary, healthy bovines (samples 26-32) do not give a signal which is significantly higher than that observed with E. coli-control cytoplasm.

Antibodies directed against p362 are already present in the early stages of the disease (samples 1-13). p362 can thus be considered as a very suitable antigen for specific and sensitive diagnosis of paratuberculosis.

To decrease the background levels due to cross reaction with the  $\beta$ -galactosidase part of the fusion protein, the insert coding for p362 was recloned into another expression vector (pmTNF-MPH, Innogenetics) (Figures 9a and 9b).

It contains the tetracycline resistance gene and the origin of replication of pAT<sub>153</sub> (obtainable from Bioexcellence, Biores B.V., Woerden, The Netherlands), the lambda PL promoter up to the MboII site in the N gene 5' untranslated region (originating from pPL(λ); Pharmacia), followed by a synthetic ribosome binding site (see sequence data), and the information encoding the first 25 AA of mTNF (except for the initial Leu which is converted to Val). This sequence is, in turn, followed by a synthetic polylinker sequence which encodes six consecutive histidines followed by several proteolytic sites (a formic acid, CNBr, kallikrein, and E. coli protease VII sensitive site, respectively), each accessible via a different restriction enzyme which is unique for the plasmid (SmaI, NcoI, BspMII and StuI, respectively; see restriction and genetic map, Figure 9a). Downstream from the polylinker, several transcription terminators are present including the E.

coli trp terminator (synthetic) and the rrnBT<sub>1</sub>T<sub>2</sub> (originating from pKK223-3; Pharmacia). The total nucleic acid sequence of this plasmid is represented in Figure 9b.

Table 5 gives a complete restriction site analysis of pmTNF-MPH.

The presence of 6 successive histidines allows purification of the fusion protein by Immobilized Metal Ion Affinity Chromatography (IMAC).

To subclone the insert coding for p362 in pmTNF-MPH, it was set free from the construct in vector pUEX2 by EcoRI digestion. The EcoRI fragment (507 bp) was eluted from the gel, purified, blunted and inserted in the blunted XbaI site of pmTNF-MPH. The resulting recombinant plasmid, pmTNF-MPH-a362, is brought into E. coli strain K12AH (ATCC 33767) by transformation. After growth at 28°C, expression of the recombinant protein is induced by a temperature shift to 42°C, which is held on during 2 hours. Cells were harvested, centrifuged and lysed in French press.

The expressed fusion protein mTNF-H6-p362, present in the cytoplasm fraction of the E. coli recombinant, is purified by Immobilized Metal Ion Affinity Chromatography (IMAC) using conditions known by the man skilled in the art. The amino acid sequence of this complete fusion protein is represented in Figure 10.

The purified fusion protein is used to coat 96-well microtitration plates, which were incubated with serial dilutions of sera from uninfected (control) and infected animals. Plate bound IgG were titrated with peroxidase-labeled rabbit anti-bovine IgG, as described in Materials and Methods.

CLAIMS

1. Polypeptide containing in its polypeptidic chain:
  - the amino acid sequence of 101 amino acids of Figure 8,
  - or a fragment of this sequence, this fragment being such that:
    - . it is liable to be recognized by antibodies also recognizing the abovesaid sequence of 101 amino acids, but it is not recognized by antibodies respectively raised against M. bovis, M. avium, M. phlei and M. tuberculosis, and possibly against M. leprae, M. intracellulare, M. scrofulaceum, M. fortuitum, M. gordonae and M. smegmatis,
    - . it is liable to generate antibodies which also recognize the abovesaid sequence of 101 amino acids but which do not recognize M. bovis, M. avium, M. phlei and M. tuberculosis, and possibly M. leprae, M. intracellulare, M. scrofulaceum, M. fortuitum, M. gordonae and M. smegmatis,
    - . it reacts with the majority of sera from cattle suffering from Johne's disease,
  - or the polypeptidic sequences resulting from the modification by substitution and/or by addition and/or by deletion of one or several amino acids in so far as this modification does not alter the above-mentioned properties.
2. Polypeptide according to Claim 1, characterized by the fact that it is constituted by the sequence represented on Figure 8, extending from the extremity constituted by amino acid at position (1) to the extremity constituted by amino acid at position (101), or by the following peptides:  
Glu-Phe-Pro-Gly-Gly-Gln-Gln-His-Ser-Pro-Gln,

Gln-Gln-Ser-Tyr-Gly-Gln-Glu-Pro-Ser-Ser-Pro-Ser-Gly-Pro-Thr-Pro-Ala.

3. Polypeptide according to Claim 1, characterized by the fact that:

- it contains the amino sequence of 101 amino acids of Figure 8 as its C-terminal part,
- it has a molecular weight of about 34kDa, in SDS-PAGE,
- it is coded by a nucleotide sequence liable to hybridize with the complementary strand of the sequence of Figure 11,
- it reacts with the majority of sera from cattle suffering from Johne's disease,
- it is advantageously liable to elicit a cellular immune response in sensitized subjects.

4. Amino acid sequences constituted by anyone of the polypeptides according to Claims 1 to 3 and a protein or an heterologous sequence with respect to said polypeptide, said protein or heterologous sequence comprising for instance from about 1 to about 1100 amino acids.

5. Nucleic acid characterized by the fact that it comprises or is constituted by:

- a nucleotide chain liable to hybridize with the nucleotide chain coding for the polypeptides according to anyone of Claims 1 to 3, or
- a nucleotide chain coding for the polypeptides according to anyone of Claims 1 to 3, or
- the complementary sequences of the above nucleotide chains.

6. Nucleic acid according to Claim 5, characterized by the fact that it comprises or is constituted by a nucleotide chain,

- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (307) on Figure 7A, or

- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (508) on Figure 7A, wherein
- X and E represent phosphodiester bonds, Y and F represent respectively G and C, Z and H represent respectively C and G,  
or
- X and E represent respectively G and C, Y and F represent respectively C and G, Z and H represent phosphodiester bonds.

7. Nucleic acid according to Claim 5, characterized by the fact that it comprises or is constituted by a nucleotide chain,

- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (306) on Figure 7C, or
- extending from the extremity constituted by nucleotide at position (1) to the extremity constituted by nucleotide at position (507) on Figure 7C.

8. Nucleic acid according to Claim 5, which comprises or is constituted by:

- a nucleotide sequence liable to hybridize with the complementary strand of the nucleotide sequence of Figure 11, or with the complementary strand of the nucleotide sequence extending from nucleotide at position (742) to nucleotide at position (1338) of Figure 11,
- the nucleotide sequence of Figure 11 or the nucleotide sequence extending from nucleotide at position (742) to nucleotide at position (1338) of Figure 11,
- the complementary sequences to the above-defined sequences.

9. Recombinant nucleic acid containing at least one of the nucleotide sequences of anyone of Claims 5

to 8 combined to or inserted in a heterologous nucleic acid.

10. Recombinant vector particularly for cloning and/or expression, comprising a vector sequence, notably of the type plasmid, cosmid, phage or virus DNA and a recombinant nucleic acid according to anyone of Claims 5 to 8, inserted in one of the non essential sites for its replication.

11. Recombinant vector according to Claim 10, containing necessary elements to promote the expression in a cellular host of polypeptides coded by nucleic acids according to anyone of Claims 5 to 8 inserted in said vector and notably a promoter recognized by the RNA polymerase of the cellular host, particularly an inducible promoter and possibly a sequence coding for transcription termination and possibly a signal sequence and/or an anchoring sequence.

12. Recombinant vector according to Claim 10, containing the elements enabling the expression by E. coli of a fusion protein consisting of the polypeptide of  $\beta$ -galactosidase or part thereof linked to a polypeptide coded by a nucleic acid according to anyone of Claims 5 or 8.

13. Cellular host chosen from among bacteria such as E. coli or chosen from among eukaryotic organisms, such as CHO cells or insect cells, which is transformed by a recombinant vector according to anyone of Claims 9 to 12, and containing the regulation elements enabling the expression of the nucleotide sequence coding for the polypeptide according to anyone of Claims 1 to 3 in this host.

14. Expression product of a nucleic acid expressed by a transformed cellular host according to Claim 13.

15. Antibody characterized by the fact that it is specifically directed against a polypeptide according to anyone of Claims 1 to 3, and preferably by the fact

that it recognizes neither M. bovis, nor M. avium, nor M. phlei, nor M. tuberculosis.

16. Process for preparing a recombinant polypeptide according to anyone of Claims 1 to 4 comprising the following steps:

- the culture in an appropriate medium of a cellular host which has previously been transformed by an appropriate vector containing a nucleic acid according to anyone of Claims 5 to 8, and
- the recovery of the polypeptide produced by the above said transformed cellular host from the above said culture medium or from the cellular host.

17. Method for the in vitro diagnosis of paratuberculosis in an animal liable to be infected by Mycobacterium paratuberculosis comprising

- contacting a biological sample taken from an animal with a polypeptide according to anyone of Claims 1 to 3, or the expression product according to Claim 14, under conditions enabling an in vitro immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has been possibly formed.

18. Method for the in vitro diagnosis of paratuberculosis in an animal liable to be infected by M. paratuberculosis, comprising the following steps:

- contacting a biological sample with an appropriate antibody according to Claim 15, under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. paratuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

19. Method for the in vitro diagnosis of Crohn's disease in a patient liable to be infected by

Mycobacterium paratuberculosis comprising the following steps:

- contacting a biological sample with an appropriate antibody according to Claim 15, under conditions enabling an in vitro immunological reaction between said antibody and the antigens of M. paratuberculosis which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which may be formed.

20. Method for the in vitro diagnosis of Crohn's disease in a patient liable to be infected by M. paratuberculosis, comprising the following steps:

- contacting a biological sample taken from a patient with a polypeptide according to anyone of Claims 1 to 3, or the expression product according to Claim 14, under conditions enabling an in vitro immunological reaction between said polypeptide and the antibodies which are possibly present in the biological sample and
- the in vitro detection of the antigen/antibody complex which has been possibly formed.

21. Necessary or kit for an in vitro diagnosis method of paratuberculosis in an animal liable to be infected by Mycobacterium paratuberculosis according to Claim 17, comprising:

- a polypeptide according to anyone of Claims 1 to 3, or the expression product of Claim 14,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned polypeptide is not labeled.

22. Necessary or kit for an in vitro diagnosis method of paratuberculosis in an animal liable to be

infected by Mycobacterium paratuberculosis according to Claim 18, comprising:

- an antibody according to Claim 15,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagents possibly having a label or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned antibody is not labeled.

23. Necessary or kit for an in vitro diagnosis method of Crohn's disease in a patient liable to be infected by Mycobacterium paratuberculosis according to Claim 19, comprising:

- an antibody according to Claim 15,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complexes which have been produced by the immunological reaction, said reagents possibly having a label or being liable to be recognized by a labeled reagent, more particularly in the case where the above mentioned antibody is not labeled.

24. Necessary or kit for an in vitro diagnosis method of Crohn's disease in a patient liable to be infected by Mycobacterium paratuberculosis according to Claim 20, comprising:

- a polypeptide according to anyone of Claims 1 to 3, or the expression product of Claim 14,
- reagents for making a medium appropriate for the immunological reaction to occur,
- reagents enabling to detect the antigen/antibody complex which has been produced by the immunological reaction, said reagents possibly having a label, or being liable to be recognized by a labeled reagent,

more particularly in the case where the above mentioned polypeptide is not labeled.

25. Immunogenic composition comprising a polypeptide according to anyone of Claims 1 to 3, or the expression product of Claim 14, in association with a pharmaceutically acceptable vehicle.

26. Vaccine composition comprising among other immunogenic principles anyone of the polypeptides according to anyone of Claims 1 to 3 or the expression product of Claim 14, possibly coupled to a natural protein or to a synthetic polypeptide having a sufficient molecular weight so that the conjugate is able to induce *in vivo* the production of antibodies neutralizing Mycobacterium paratuberculosis, or induce *in vivo* a protective cellular immune response by activating M. paratuberculosis antigen-responsive T cells.

27. Necessary or kit for the diagnosis of prior exposure of an animal to M. paratuberculosis, said necessary or kit containing a preparation of at least one of the polypeptides or peptides according to anyone of Claims 1 to 3, or the expression product of Claim 14, with said preparation being able to induce *in vivo* after being intradermally injected to an animal a delayed type hypersensitivity reaction, at the site of injection, in case the animal has had prior exposure to M. paratuberculosis.

28. Polypeptides according to claim 3, characterized in that they contain or are constituted by:

- the amino acid sequence of Figure 11 or
- the amino acid sequence extending from amino acid at position (1) to amino acid at position (199) of Figure 11.

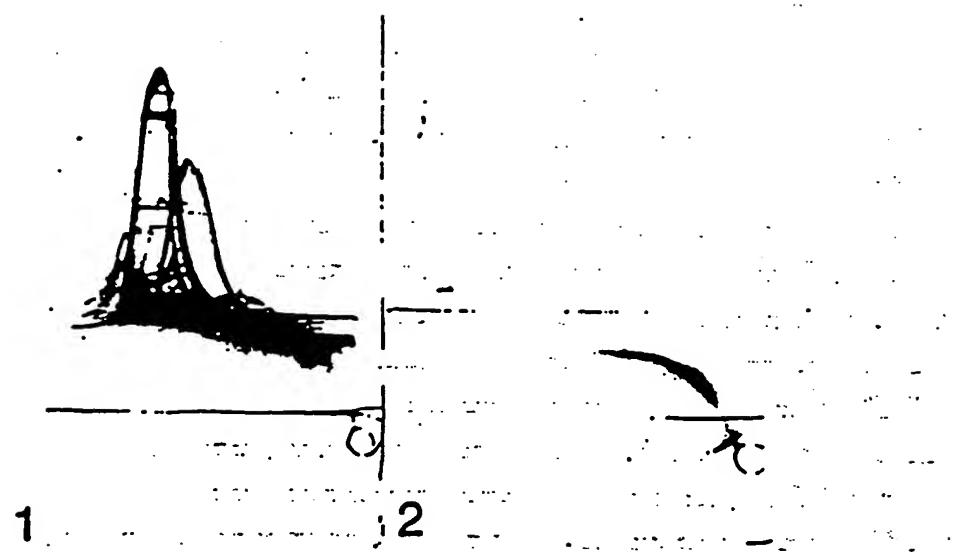
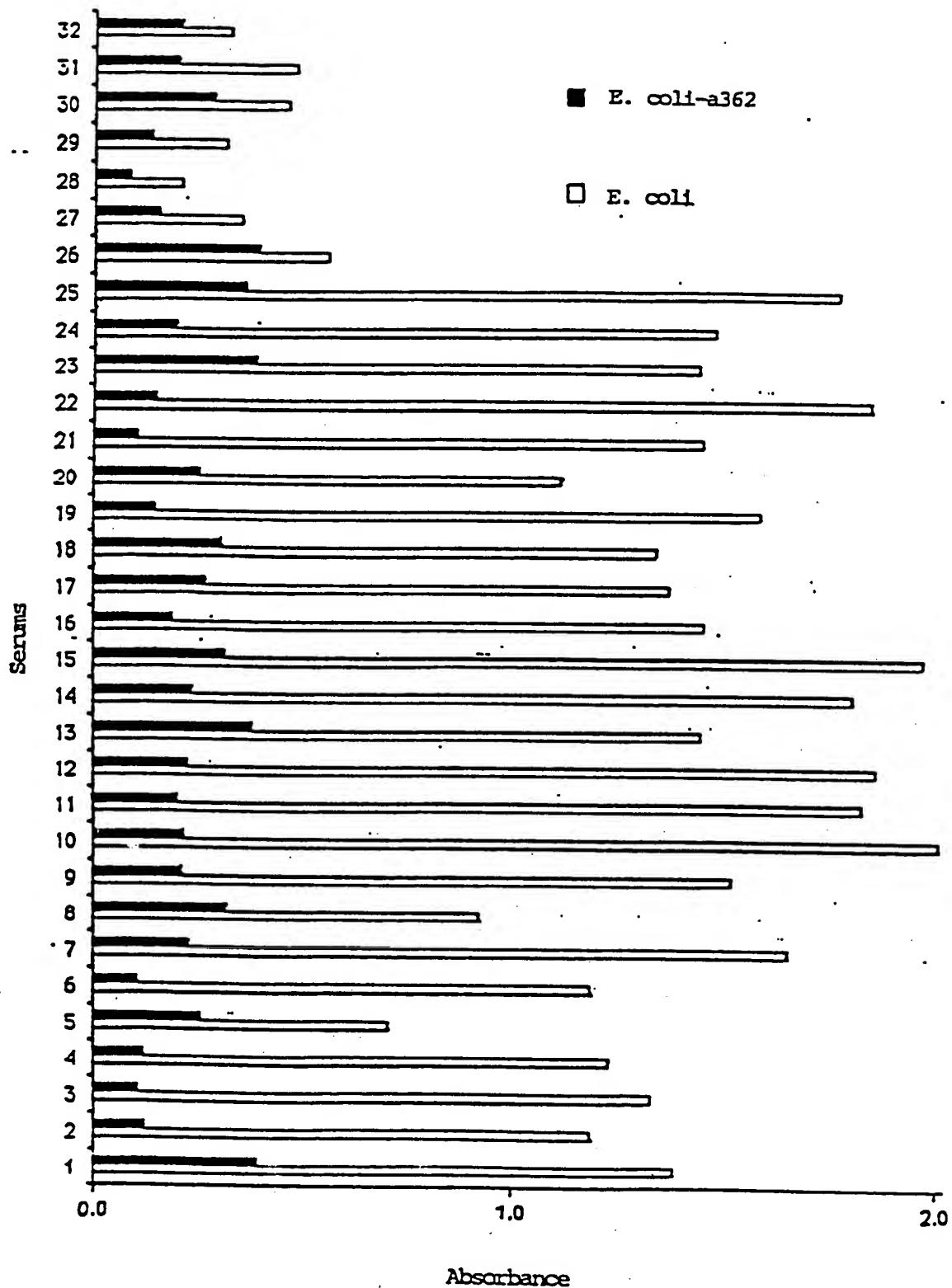


Figure 1 (1)

Figure 1 (2)

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Figure 2



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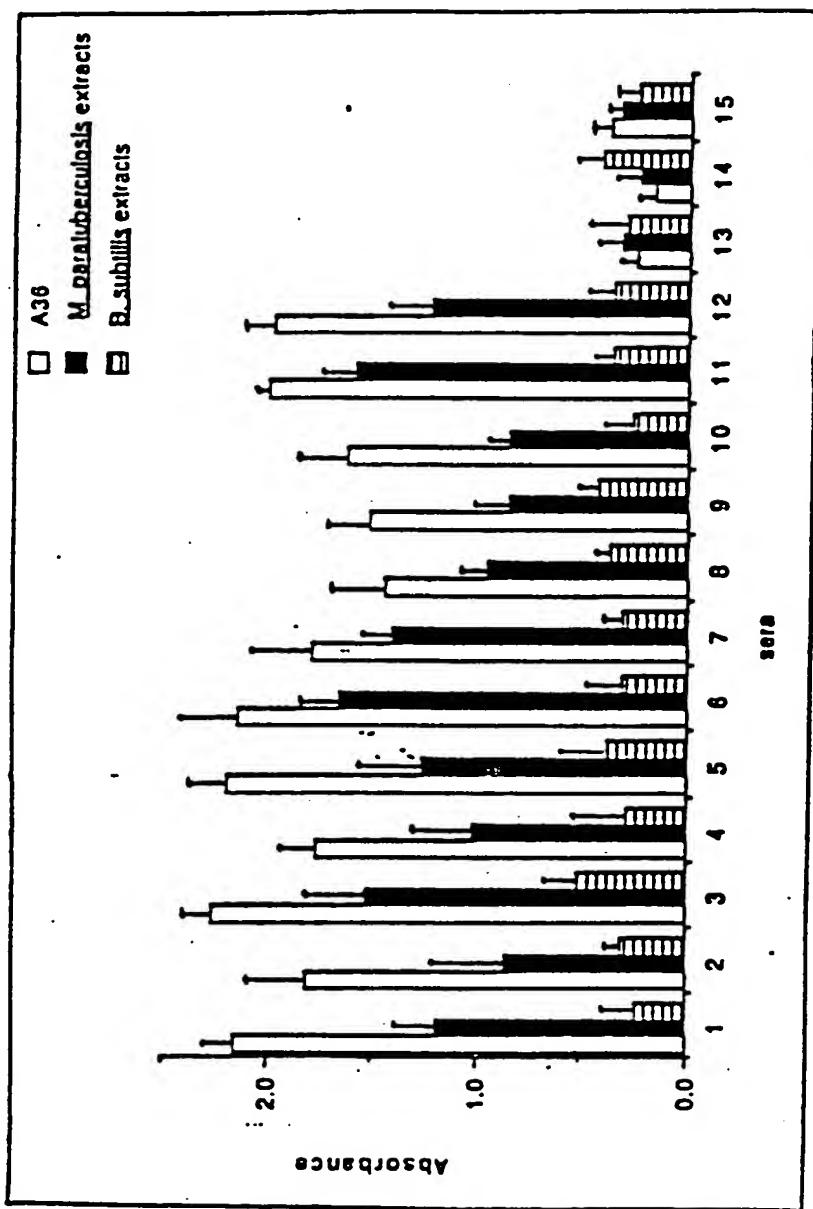


Fig. 3

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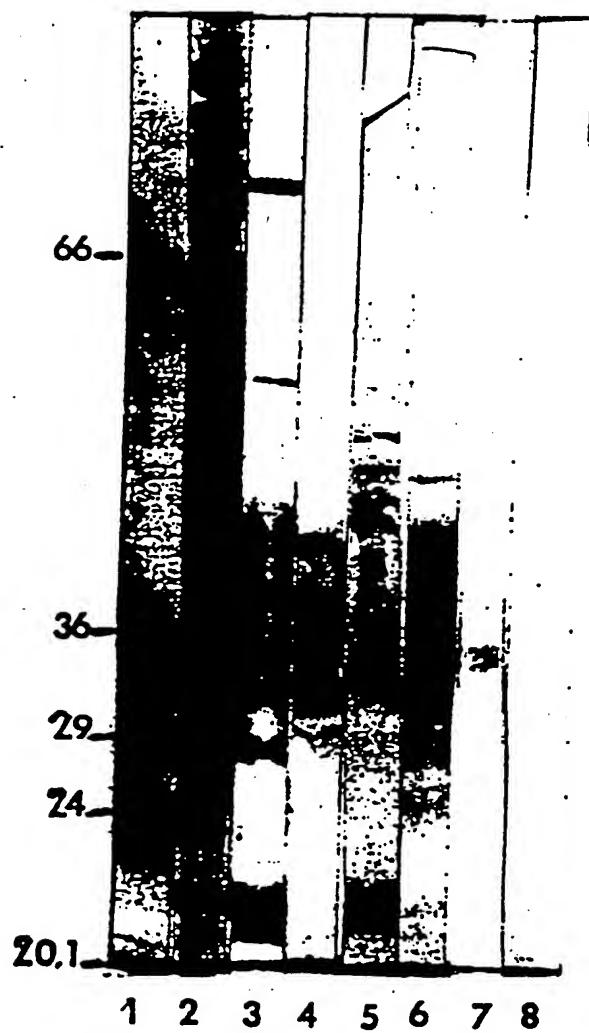


Figure 4

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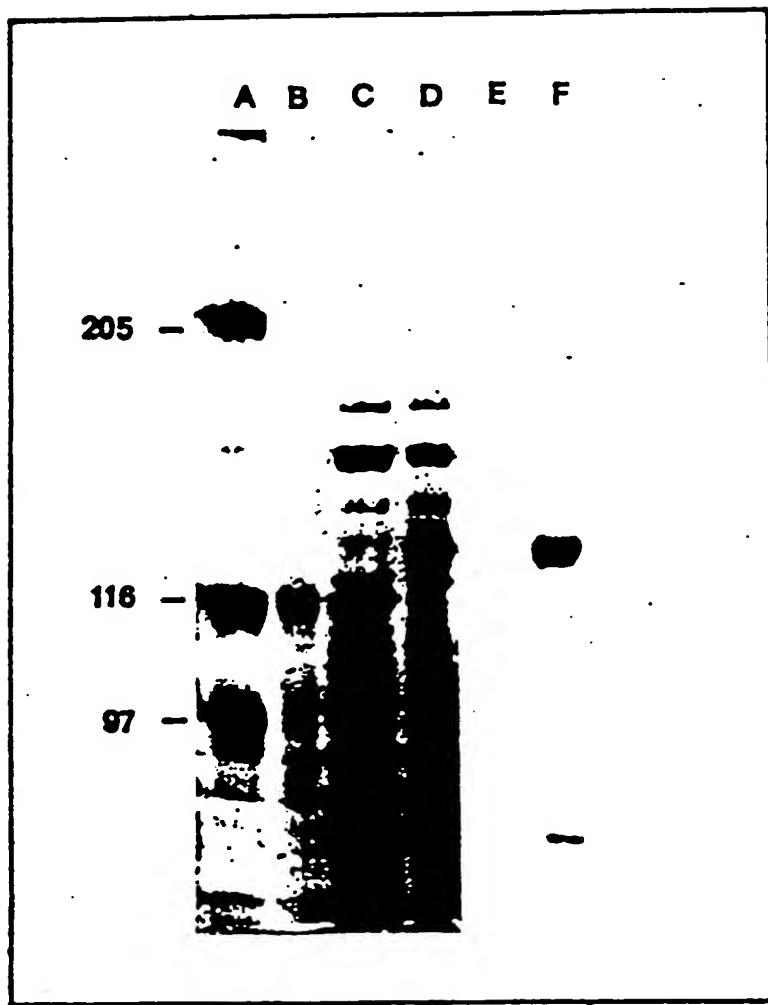


Figure 5

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Figure 6

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GAATTCCCGG GTGGTCAGCA GCATTGCCG CAGGXCTACG GGTGCGAGTA CGGGCGTTAC  
CTTAAAGGGC CACCACTCGT CGTAGGGCC GTTACGGGC GTTCEGATGC CCAGGCTCAT CGGCCAAATG 60

GGCCAGGGGG GGGCTCCGAC CGGGGGTTTC CGTGGCCAGC CGTGGCCGCA GTCCGGCCCG  
CCGGTCCCGC CGCGAGGCTG CCCGCAAAAG CCACGGGTG GCAGGGCGT CAGGGCGGC 120

CACAGTCGG CGCAGCAGCA GGGCCGTCAC ACACCGCCA CCGGCTTCCC CAGCTTCAGC  
GTTGTCAGGC CGGTGCTGTT CCCGGCAGG TGTGGGGT GGCGGAAGGG GTCGAAGTTCG 180

CCGXYGCCCA ACGTGGGGG GGGATGGAC TCCGGTTCCG CGAACGCCAA TTACTCCGAG  
GGCGPCGGGT TGCAGCCGCC CCCTAGGCTG AGGCCAAGGC GCTGGGGTT AATGAGGGCTC 240

CAGGGCGGTG GZCAGGAGT CCTAAGGGCA GGAGGCTCT TCACCGCTTG GGGCACGCC  
GTTCGGCCAC CHGGTCTGCA GGATGCCGGT CCTCGGAAGA AGTGGCAGAC CGGGCTGGGG 300

CGCCTAAGT GCCCTGTGCG GCCTAGTGG GAACGTGCC CAGAGTGACA CGGGTGGAGG  
GGGGATTGCA CGGGACAGGG CGGATCAGGC CTGCAAGGG GTCTCACGT GCCCACCTCC 360

ACAAACCGGGC AGGGGGGCT CGCCAGGGCC GTGACCTCGT CAGGGTGGCG TTTCGGCCGG  
TGTGGCCCG TGGCCCCGA GGGTCCGGC CACTGGAGCA GTCCCCAGGCC AAGGGGGCC 420

CGGTGGTGGC ACTGGTCATC ATGGCCGGG TCACGGCTGAT CCAGTTGTTG ATCGCCAACA  
GCCACCACCG TGACCCAGTAG TAGGGGGCC AGTGGGACTA GGTCAAACAC TAGGGGTTGT 480

GGGACATGAC CGGGGGGTTG GGGAAATTC  
CCCTGTACTG GCCGGGCAAC CCCTTAAG 508

Fig. 7A

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GAATTCCCGG	GTGGTCAGCA	GCATTCGGG	CAGGCTACGG	GTGCCAAGTAC	GGCGGTTACG	60
CTTAAGGGCC	CACCAAGTCGT	CGTAACGGG	GTCCGATGCC	CAGCGTCATG	CGCCAATATGC	
GGCAGGGGG	CGCTTCGACC	GGGGGTTTCG	GTGCCCAAGCC	GTGCCGCCAG	TCCGGCCCGC	120
CGGTCCCCC	GGGAGGCTGG	CCGCCAAAGC	CACGGGTCCG	CAGGGGTTC	AGGCGGGCGG	
AACAGTCGG	GGAGGAGCAG	GGCCCCTCCA	CACCGCCCAC	GGGTTCCCC	AGCTTCAGCC	180
TTGTCAAGGG	CGTCGTCGTC	CCGGGCAGGT	GTGGGGTGTG	GCGGAAGGG	TCGAAGTCGG	
GGCGGGCCAA	CGTCGGGGG	GGATGGACT	CGGTTTGGC	GACCGCCAAT	TACTCCGAGC	240
GGGGCGGGTT	GCAGCCGCC	CCTAGCCCTGA	GGCCAAGGCCG	CTGGGGTATA	ATGAGGCTCG	
AGCCGGGG	CCCAAGGTC	CTACGGCCAG	GAGCCTTCTT	CACCGTCTGG	GCCGACGCC	300
TCCGGCCACC	GGGTGTCAG	GATGCCGGTC	CTCGGAAGAA	GTGGCAGACC	CGGCTGGGG	
GCCTAACGTT	CCCTGTCGG	CCTAGTCGGG	AACGTGCCCG	AGAGTGACAC	GGGTGGAGGA	360
CGGATTGCCAC	GGGACAGGGC	GGATCAGCCC	TTGCACGGGG	TCTCACTGTG	CCCACCTCCCT	
CNAACGGGCA	GGGGGGCTC	GCCAGGGGG	TGACCTCTGTC	AGGGTCGGGT	TCGGCCCCGGC	420
GTIGGGCCGT	CGCCCGGAG	CGGTCCGGCC	ACTGGAGGAG	TCCCAGGCAC	AGGGGGCGG	
GGTGGTGGCA	CTGGTCATCA	TCGGCGGGGT	CACGCTGATC	CAGITGTGTA	TCGCCAACAG	480
CCACCAACGT	GACCAAGTAGT	AGCGGGGCCA	GTGGGACTAG	GTCAAAACT	AGGGGTGTGTC	
CGACATGACC	GGGGCGTTGG	GGAAATTG				
GCTGTACTGG	CGGGCAAC	CCTTAAG				

Fig. 7B

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GAATTCCCGG	GTGGTCAGCA	GCATTCGGCG	CAGGGCTACG	GGTCCGACTA	GGGGGGTTAC	60
CTTAAGGGCC	CACCAAGTCGT	CGTAAGGGCC	GTCCCGGATGC	CCAGGGTCAT	GCGCCAAATG	
GGCCAGGGG	GGGCTCCGAC	CGGGGGTTTC	GGTCCCCAGC	CGTCCGGCGCA	GTCCGGGCCG	120
CCGGTCCCCC	CGCGAGGCTG	GGCGGCCAAG	CCACGGGTCC	GCAGGGCGT	CAGGGGGCC	
CAACAGTCCG	CGCAGGAGCA	GGGGCCCGTCC	ACACCCGCCCA	CCGGCTTCCC	CAGGTCAGC	180
GTTGTCAGGC	GGGTGTCGT	CCCCGGCAAG	TGTGGGGGT	GGCCGAAAGG	GTCGAAGTCG	
CGGCCGCCCA	ACGTGGGGG	GGGATCGGAC	TCCGGTTCGG	CGACCCGCAA	TTACTCCGAG	240
GGGGGGGGT	TGCAGCCGCC	CCCTAGGCTG	AGGCCAAGCC	GCTGGGGTT	AATGAGGCTC	
CAGGGCCGGTG	GCCAGGAGTC	CTACGGCCAG	GAGGCCCTCTT	CACCGTCTGG	GGCGACGGCC	300
GTCCGGCCAC	GGTGTTCAG	GATGCCGGTC	CTCGGAAGAA	GTGGCACACC	CGGCTGGGG	
GCCTAACGTTG	CCCTGTGGG	CCTAGTGGGG	AACTGTGCC	AGAGTGACAC	GGTGGAGGA	360
CGGATTGGAC	GGGACAGGGC	GGATCAGGGC	TTCACCGGG	TCTCATCTGTG	CCCACCTCTCT	
CAACGGGGCA	GGGGGGGCTC	GCCAGGGGG	TGACCTCGTC	AGGGTTCGGT	TCGGCCGGC	420
GTTGGCCCGT	CGCCCCGGAG	CGGTCCGGCC	ACTGGAGCAG	TCCCAGGCAC	AGGGGGCCG	
GGTGGTGGCA	CTGGTCATCA	TGGCCGGGT	CACGGCTGATC	CAGTTGTTGA	TCGCCAACACG	480
CCACCAACGT	GACCAGTAGT	AGGGGGCCA	GTGCGACTAG	GTCAACAACT	AGGGGTTGTC	
CGACATGACC	GGGGGGTTGG	GGAAATTG				
GCTGTACTGG	CGGGCAACCC	CCTTAAG				

Fig. 7C

10/27

GAA TTC CCG GGT GGT CAG CAG CAT TCG CCG CAG GGC TAC GGG TCG  
 Glu Phe Pro Gly Gly Gln Gln His Ser Pro Gln Gly Tyr Gly Ser  
 5                   10                   15

CAG TAC GGC GGT TAC GGC CAG GGC GGC GCT CCG ACC GGC GGT TTC  
 Gln Tyr Gly Gly Tyr Gly Gln Gly Gly Ala Pro Thr Gly Gly Phe  
                  20                 25                 30

GGT GCC CAG CCG TCG CCG CAG TCC GGC CCG CAA CAG TCC GCG CAG 135  
 Gly Ala Gln Pro Ser Pro Gln Ser Gly Pro Gln Gln Ser Ala Gln  
 35 40 45

CAG CAG GGC CCG TCC ACA CCG CCC ACC GGC TTC CCC AGC TTC AGC 180  
 Gln Gln Gly Pro Ser Thr Pro Pro Thr Gly Phe Pro Ser Phe Ser  
                   50                  55                  60

CCG CCG CCC AAC GTC GGC GGG GGA TCG GAC TCC GGT TCG GCG ACC 225  
 Pro Pro Pro Asn Val Gly Gly Ser Asp Ser Gly Ser Ala Thr  
       65              70              75

GAG CCT TCT TCA CCG TCT GGG CCG ACG CCC GCC TAA  
 Glu Pro Ser Ser Pro Ser Gly Pro Thr Pro Ala ---  
                   95                  100

Fig. 8

11/27

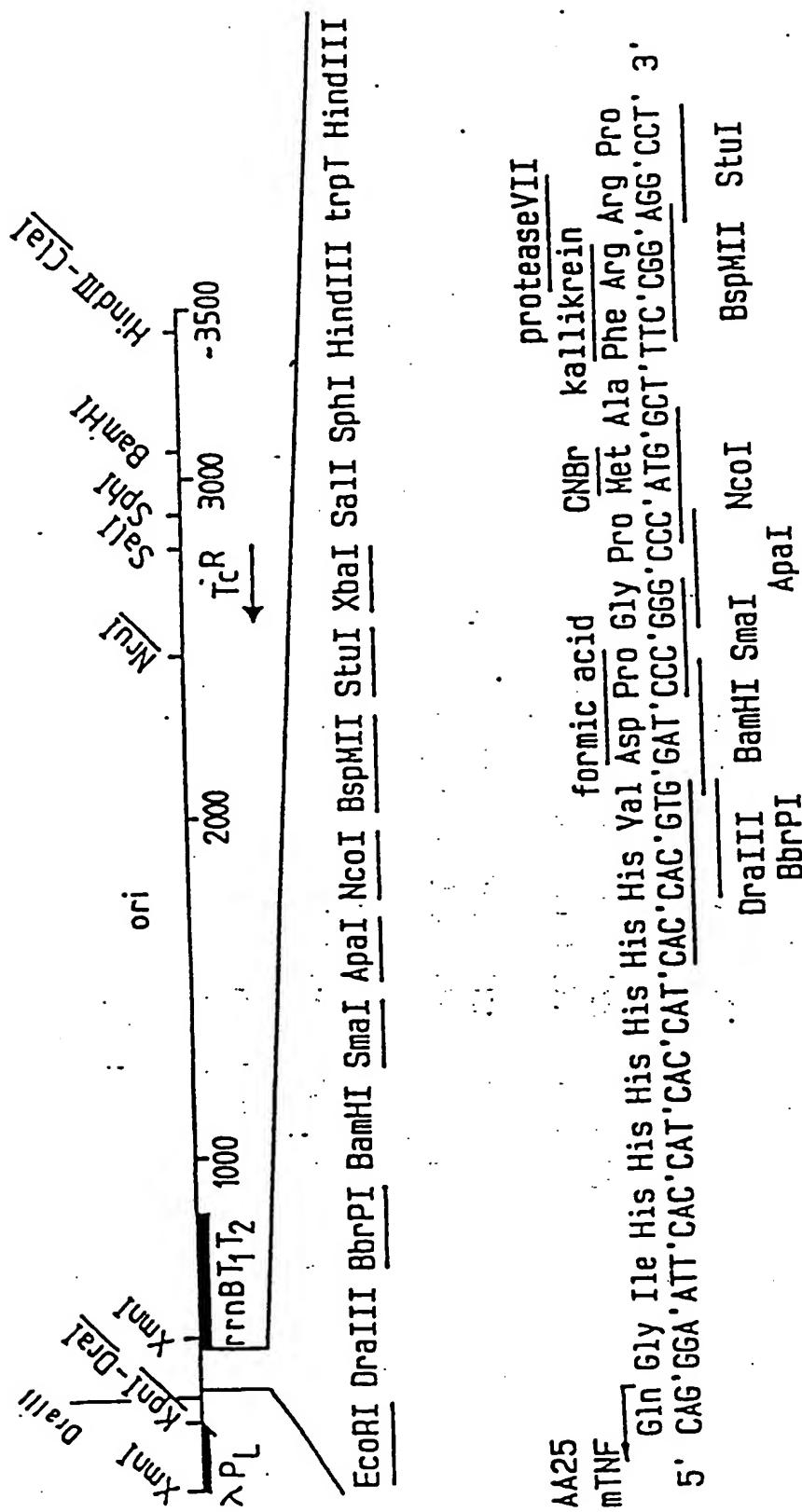


Figure 9a

12/27

From:	pmTNF mRNA	3	9	15	21	27	33	39	45
1	AAT TCC GGG GAT CTC TCA CCA ACC MAA CAA TGC CCC CCT GCA CGT TAA	-	-	-	-	-	-	-	-
1	TAA AGG CCC C'TA GAG AGT GGA TGG TTT GTT ACG GGG GGA CGT T'IT	-	-	-	-	-	-	-	-
46	AAT AAA TTC ATA TAA MAA MCA TAC AGA TAA CCA TCT GCG GTG ATA	-	-	-	-	-	-	-	-
	TTA TTT AAG TAT ATT TTT TGT ATG TCT ATT GGT AGA CGC CAC TAT	-	-	-	-	-	-	-	-
91	AAT TAT CTC TGG CGG TGT TGA CAT AAA TAC CAC TGG CGG TGA TAC	-	-	-	-	-	-	-	-
	TTA ATA GAG ACC GCC ACA ACT GTA ATT ATG GTG ACC GCC ACT ATG	-	-	-	-	-	-	-	-
136	TGA GCA CAT CAG CAG GAC GCA CTG ACC ACC ATG AAG GTG ACG CTC	-	-	-	-	-	-	-	-
	ACT CGT GTA GTC GTC CTG CGT GAC TGG TAC T'IC CAC TGC GAG	-	-	-	-	-	-	-	-
181	TTA AAA ATT AAG CCC TGA AGA AGG GCA GGG GTA CCA GGA GGT TTA	-	-	-	-	-	-	-	-
	AAT TTT TAA 'RTC GGG ACT TCT TCC CGT CCC CAT GGT CCT CCA ATT	-	-	-	-	-	-	-	-
226	AAT CAT GGT AAG ATC AAG TAG TCA AAA TTC GAG TGA CAA GCC TGT	-	-	-	-	-	-	-	-
	TTA GTA CCA TTC TAG TTC ATC AGT TTT AAG CTC ACT GTT CGG ACA	-	-	-	-	-	-	-	-
271	AGC CCA CGT CGT AGC MAA CCA CCA AGT GGA GGA GGG ATT TCA	-	-	-	-	-	-	-	-
	TCC GGT GCA GCA TCG TTT GGT GGT TCA CCT CGT CCC TTA AGT	-	-	-	-	-	-	-	-
316	CCA TCA CCA TCA CGT GGA TCC CGG GCC CAT GGC TTT CCG GAG	-	-	-	-	-	-	-	-
	GGT AGT GGT AGT GGT GCA CCT AGG GCC CGG GTA CCC AAA GGC CTC	-	-	-	-	-	-	-	-

Figure 9b

13/27

361	CCC	TCT	AGA	GTC	GAC	CGC	CAT	GCA	AGC	TTA	AGT	AGG	TAA	GCC	GCC	
	CGG	AGA	TCT	CAG	CTG	GCC	GTG	CGT	TCG	AAT	TCA	TTC	ATT	CGG	CGG	
406	AGT	TCC	GCT	GGC	GGC	ATT	TTN	NTT	GAT	GGC	CAA	GCT	TGG	CTG	TTT	
	TCA	AGG	CGA	CCG	CCG	TAA	AAN	NAA	CTA	CGG	GTT	CGA	ACC	GAC	AAA	
451	TGG	CGG	ATG	AGA	GAA	GAT	TTT	CAG	CCT	GAT	ACA	GAT	TAA	ATC	AGA	
	ACC	GGC	TAC	TCT	CTT	CTA	AAA	GTC	GGG	CTA	TGT	CTA	ATT	TAG	TCT	
496	ACG	CAG	AGG	CGG	TCT	GAT	AAA	ACA	GAA	TTT	GGC	TGG	CGG	CAG	TAG	
	TGC	GTC	TTC	GGC	AGA	CTA	TTT	TGT	CTT	AAG	CGG	ACC	GGC	GTC	ATC	
541	CGC	GGT	CCC	ACC	TGA	CCC	CAT	GGC	GAA	CTC	AGA	AGT	GAA	ACG		
	GGG	CCA	GGG	TGG	ACT	GGG	GTG	GGG	CTT	GAG	TCT	TCA	CTT	TGC		
586	CCG	TAG	CGC	TGG	TAG	TCT	GGG	GTC	TCC	CCA	TGC	GAG	AGT	AGG		
	GGC	ATC	GCG	GCT	ACC	ATC	ATC	ACG	CCC	CAG	AGG	GGT	ACG	CTC	TCC	
631	GAA	CTG	CCA	GGC	ATC	AAA	TAA	AAC	GAA	AGG	CTC	AGT	CGA	AAG	ACT	
	CTT	GAC	GGT	CCG	TAG	TTT	ATT	TTG	CTT	TCC	GAG	TCA	GCT	TTC	TGA	
676	GGG	CCT	TTT	GTT	TCT	TCT	GTT	TGT	CGG	TGA	ACG	CTC	TCC	TGA		
	CCC	GGA	AAG	CAA	AAT	AGA	CAA	ACA	GGC	ACT	TGC	GAG	AGG	ACT		
721	GTA	GGG	CAA	ATC	CGC	CGG	GAG	CGG	ATT	TGA	ACG	TTG	CGA	AGC	AAC	
	CAT	CCT	GTT	GGT	TAG	GGG	GGC	CTC	GCC	TAA, <sub>t</sub> Figure 9b (con't)	ACT	TGC	AAC	GCT	TCG	TTG

14/27

766	GGC	CCG	GAG	GGT	GGC	GGG	CAG	GAC	GCC	CGC	CAT	AAA	C'TG	CCA	GGC
	CCG	GGC	CTC	CCA	CCG	CCC	GTC	CTG	GGG	GCG	GTA	TAA	GAC	GGT	CCG
811	ATC	AAA	TAA	AGC	AGA	AGG	CCA	TCC	TGA	CGG	ATG	GCC	T'TT	TTG	CGT
	TAG	T'TT	AAT	TCC	TCT	TCC	GGT	AGG	ACT	GCC	T'AC	CGG	AAA	AAC	GCA
856	T'TC	TAC	AAA	CTC	TTT	TGT	TTA	TTT	TTC	TAA	ATA	CAT	TCA	AAT	ATG
	AAG	ATG	T'TT	GAG	AAA	ACA	ATT	AAA	AAG	ATT	TAT	TAT	GTA	AGT	TTA
901	TAT	CCG	C'TC	ATG	AGA	CMA	TAA	CCC	TGA	TAA	ATG	C'TT	CAA	TAA	TAA
	ATA	GGC	GAG	TAC	TCT	G'IT	ATT	GGG	ACT	ATT	'TAC	GAA	GTT	ATT	ATT
946	AAG	GAT	CTA	GGT	GAA	GAT	CCT	TTT	TGA	TAA	TCT	CAT	GAC	CMA	ATT
	TTC	CTA	GAT	CCA	CTT	CTA	GGA	AAA	ACT	ATT	AGA	GTA	CTG	GTT	TTA
991	CCC	TTA	ACG	TGA	GTT	TTC	GTT	CCA	CTG	AGC	GTC	AGA	CCC	CGT	AGA
	GGG	AAT	TGC	ACT	CAA	AAG	CAA	GGT	GAC	TCG	CAG	TCT	GGG	GCA	TCT
1036	AAA	GAT	CAA	AGG	ATC	TTC	TTC	AGA	TCC	T'TT	TTT	TCT	GCG	CGT	ATT
	T'TT	CTA	GTT	TCC	TAG	AAG	AAC	TCT	AGG	AAA	AAA	AGA	CGC	GCA	TTA

Figure 9b (con't)

1081 CTG CTG CTT GCA AAC AAA ACC ACC GCT ACC AGC GGT GGT TTG  
 GAC GAC GAA CGT TTG TTT TGG CGA TGG TCG CCA AAC  
  
 1126 TTT GCC GGA TCA AGA GCT ACC AAC TCT TTT TCC GAA GGT AAC TGG  
 AAA CGG CCT AGT TCT CGA TGG TTG AGA AAA AGG CTT CCA TTG ACC  
  
 1171 CTT CAG CAG AGC GCA GAT ACC AAA TAC TGT CCT TCT AGT GTA GCC  
 GAA GTC GTC TCG CCG CTA TGG TTT ATG ACA GGA AGA TCA CAT CGG  
  
 1216 GTA GTT AGG CCA CCA CTT CAA GAA CTC TGT AGC ACC GCC TAC ATA  
 CAT CAA TCC GGT GGT GAA GTT CTT GAG ACA TCG TGG CGG ATG TAT<sup>15/27</sup>  
  
 1261 CCT CGC TCT GCT AAT CCT GTT ACC AGT GGC TGC TGC CAG TGG CGA  
 GGA GCG AGA CGA TTA GGA CAA TGG TCA CCG ACG ACG GTC ACC GCT  
  
 1306 TAA GTC GTG TCT TAC CGG GTT GGA CTC AAG ACG ATA GTT ACC GGA  
 ATT CAG CAC AGA ATG GCC CAA CCT GAG TTC TGC TAT CAA TGG CCT  
  
 1351 TAA GGC GCA GCG GTC GGG CTG AAC GGG GGG TTC GTG CAC ACA GCC  
 ATT CCG CGT CGC CAG CCC GAC TTG CCC CCC AAG CAC GTG TGT CGG

Figure 9b (con't)

1396 CAG CTT GGA GCG AAC GAC CTA CAC CGA ACT GAG ATA CCT ACA GCC  
GTC GAA CCT CGC TTG CTG GAT GTG GCT TGA CTC TAT GGA TGT CGC 16/27

1441 TGA GCA TTG AGA AAG CCG CAC GCT TCC CGA AGG GCT TCC CGA AAA GGC GGA  
ACT CGT AAC TCT TTC GCG GTG CGA AGG GCT TCC CGA AAA GGC GGA

1486 CAG GTA TCC GGT AAG CGG CAG GGT CGG AAC AGG AGA GCG CAC GAG  
GTC CAT AGG CCA TTC GCC GTC CCA GCC TTG TCC TCT CGC GTG CTC

1531 GGA GCT TCC AGG GGG AAA CGC CTG GTA TCT TTA TAG TCC TGT CGG  
CCT CGA AGG TCC CCC TTT GCG GAC CAT AGA AAT ATC AGG ACA GCC

1576 GTT TCG CCA CCT CTG ACT TGA GCG TCG ATT TTT GTG ATG CTC GTC  
CAA AGC GGT GGA GAC TGA ACT CGC AGC TAA AAA CAC TAC GAG CAG

Figure 9b (con't)

17/27

1621	AGG	GGG	GCG	GAG	CCT	ATG	GAA	AAA	CCG	CAG	CAG	GGC	CTT	TIT
TCC	CCC	CGC	CTC	GGG	TAC	CTT	TTT	GGG	GTC	GTC	GTC	GGG	CGG	GAA
1666	ACG	GTT	CCT	GGG	CTT	TTG	CTG	GCC	TTT	TGC	TCA	CAT	GTT	CTT
TGC	CAA	GGG	CCG	GAA	AAC	GAC	GGG	AAA	ACG	AGT	GTA	CNA	GAA	AGG
1711	TGC	GTT	ATC	CCC	TGA	TTC	TGT	GGA	TAA	CCG	TAT	TAC	CGC	CTT
ACG	CAA	TAG	GGG	ACT	AAG	ACA	CCT	ATT	GGC	ATA	ATG	GGG	GAA	ACT
1756	GTC	AGC	TGA	TAC	CGC	TCG	CCG	CAG	CCG	MAC	GAC	CAG	GCG	CGA
CAC	TCG	ACT	ATG	GGC	AGC	GGC	GTC	GGC	TTG	CTG	GCT	CGC	GTC	GCT
1801	GTC	AGT	GAG	CGA	GGA	AGC	GGA	AGA	GCG	CTG	ACT	TCC	GCG	TTT
CAG	TCA	CTC	GCT	CCT	TCG	CCT	TCT	CGC	GAC	TGA	AGG	CGC	AAA	GGT
1846	GAC	TTT	ACG	AAA	CAC	GGA	AAC	CGA	AGA	CCA	TTC	ATG	TTG	TTG
CTG	AAA	TGC	TTT	GTG	CCT	TTG	GCT	TCT	GGT	AAG	TAC	AAC	AAC	GAG
1891	AGG	TCG	CAG	ACG	TTT	TGC	AGC	AGC	AGT	CGC	TTC	ACG	TTC	GCT
TCC	AGC	GTC	TGC	AAA	ACG	TCG	TCG	TCA	GCG	AAG	TGC	AAG	CGA	CGC

Figure 9b (con't)

18/27

1936	GTA	TCG	GTG	ATT	CAT	TCT	GCT	AAC	CAG	TAA	GGC	AAC	CCC	GCC	AGC
	CAT	ACC	CAC	TAA	GTA	AGA	CGA	TTG	GTC	ATT	CCG	TTG	GGG	CGG	TCG
1981	CTA	GCC	GGG	TCC	TCA	ACG	ACA	GGA	GCA	CCT	CGT	TGC	GCA	CCC	GTG
	GAT	CGG	CCC	AGG	AGT	TGC	TGT	CGT	GCT	AGT	ACG	CGT	GGG	CAC	
2026	GCC	AGG	ACC	CAA	CGG	TGC	CCG	AGA	TGC	GCC	GCG	TGC	GGC	TGC	TGG
	CGG	TCC	TGG	GTT	GGG	ACG	GGC	TCT	ACG	CGG	CGC	ACG	CCG	ACG	ACC
2071	AGA	TGG	CGG	ACG	CGA	TGG	ATA	TGT	TCT	GCC	AAG	GGT	TGG	TTT	GCG
	TCT	ACC	GCC	TGC	GCT	ACC	TAT	ACA	AGA	CGG	TTC	CCA	ACC	AAA	CGC
2116	CAT	TCA	CAG	TTC	TCC	GCA	AGA	ATT	GAT	TGG	CTC	CAA	TTC	TTG	GAG
	GTA	AGT	GTC	AAG	AGG	CGT	TCT	TAA	CTA	ACC	GAG	GTT	AAG	AAC	CTC
2161	TGG	TGA	ATC	CGT	TAG	CGA	GGT	GGC	GGC	TTC	CAT	TCA	GGT	CGA	
	ACC	ACT	TAG	GCA	ATC	GCT	CCA	CGG	CGG	CGG	AAG	GTA	AGT	CCA	GCT
2206	GGT	GGC	CCG	GCT	CCA	TGC	ACC	GCG	ACG	CAN	CGC	GGG	GAG	GCA	GAC
	CCA	CCG	GGC	CGA	GGT	ACG	TGG	CGC	TGC	GTT	GGC	CCC	CTC	CGT	CTG

Figure 9b (con't)

19/27

2251	AAG	GTA	TAG	GGC	GGC	TAC	AAT	CCA	TGC	CAA	CCC	GTT	CCA	TGT
	TTC	CAT	ATC	CCG	CCG	ATG	TTA	GCT	ACG	GTG	GGG	CAA	GGT	ACA
2296	GCT	CGC	CGA	GGC	GGC	ATA	AAT	GGC	CGT	GAC	CAG	CGG	TCC	AGT
	CGA	GGC	GCT	CCG	CCG	TAT	TTA	GCG	GCA	CTG	CTA	GTC	GCC	AGG
2341	GAT	CGA	AGT	TAG	GCT	GGT	AAG	AGC	GGC	GAG	CGA	TCC	TTG	AAG
	CTA	GCT	TCA	ATC	CGA	CCA	TTC	TCC	GGG	CTC	GCT	AGG	AAC	TTC
2386	TCC	CTG	ATG	GTC	GTC	ATC	TAC	CTG	CCT	GGA	CAG	CAT	GGC	CTG
	AGG	GAC	TAC	CAG	CAG	TAG	ATG	GAC	GGA	CCT	GTC	GTA	CCG	GAC
2431	CGC	GGG	CAT	CCC	GAT	GGC	GGC	GGA	GGC	GAG	AAG	AAT	CAT	AAT
	CGC	CCC	GTA	GGG	CTA	CGG	CGG	CCT	TCG	CTC	TTC	TTA	GTA	TTA

figure 9b (con't)

20/27

2476	GAA	GGC	CAT	CCA	GCC	TCC	CGT	CCC	GAA	CAG	CGC	CAG	CM	GAC	GTA	GCC	
	CTT	CCG	GTA	GGT	CGG	AGC	GG	CCG	CAT	GG	GTC	GG	GTC	GTT	CTG	CAT	CGG
2521	CAG	CCC	GTC	GGC	CGC	CAT	GGC	GAT	AAT	GGC	CTG	CTT	CTC	CCC			
	GTC	GGC	CAG	CCG	GGG	GT	GGG	CTA	TTA	CCG	GAC	GAA	GAG	GAG			
2566	GAA	ACG	TTT	GCT	GGC	GGG	ACC	AGT	GAC	GAA	GGC	TTG	AGC	GAG	GGC		
	CTT	TGC	AAA	CCA	CCC	CCC	TGG	TCA	CTG	CTT	CCG	AAC	TCG	CTC	CCC		
2611	GTG	CAA	GAT	TCC	GAA	TAC	CGC	AAG	CGA	CAG	GGC	GAT	CAT	CGT	CGC		
	CAC	GTT	CTA	AGG	CTT	ATG	GGG	TTC	GCT	GTC	CGG	CTA	GTA	GCA	GCG		
2656	GCT	CCA	GGG	AAA	GGG	GTC	CTC	GGC	GAA	AAT	GAC	CCA	GAG	GGC	TGC		
	CGA	GCT	GGC	TTT	CCG	CAG	GAG	GGG	CTT	TTA	CTG	GCT	CTC	GGG	ACG		
2701	CGG	CAC	CTG	TCC	TAC	GAG	TTG	CAT	GAT	AAA	GAA	GAC	AGT	CAT	AAC		
	CCC	GTG	GAC	AGG	ATG	CTC	AAC	GTA	CTA	TTT	CTT	CTG	TCA	GTA	TTC		
2746	TGC	GCC	GAC	GAT	AGT	CAT	GCC	CCG	CCG	CCA	CCG	GAA	GG	GCT	GAC		
	ACG	CCG	CTG	CTA	TCA	GTA	GGG	GGG	GGG	GGT	GGC	CTT	CCT	CGA	CTG		

Figure 9, (continued)

21/27

2791	TGG	GTT	GAA	GGC	TCT	CAA	GGG	CAT	CGG	TCG	ACG	CTC	TCC	CTT	ATG
	ACC	CAA	CTT	CCG	AGA	GTT	CCC	GTA	GCC	AGC	TGC	GAG	AGG	GAA	TAC
2836	CGA	CTC	CTG	CAT	TAG	GAA	GCA	GCC	CAG	TAG	TAG	GTT	GAG	GCC	GTT
	GCT	GAG	GAC	GTA	ATC	CTT	CGT	CGG	GTC	ATC	ATC	CAA	CTC	CGG	CAA
2881	GAG	CAC	CGC	CGC	CGC	MAG	GMA	TGG	TGG	ATG	CMA	GGA	GAT	GCC	GCC
	CTC	GTG	GGG	GGG	GGG	TTC	CTT	ACC	ACG	TAC	GTT	CCT	CTA	CCG	CGG
2926	CAA	CAG	TCC	CCC	GGC	CAC	GGC	GGC	TGG	CAC	CAT	ACC	CAC	GCC	GAA
	GTT	GTC	AGG	GGG	CCG	GTG	CCC	GGG	ACG	GTG	GTA	TGG	GTG	CGG	CTT
2971	ACA	AGC	GCT	CAT	GAG	CCC	GAA	GTG	GCG	AGC	CCG	ATC	TTC	CCC	ATC
	TGT	TCG	CGA	GTA	CTC	GGG	CTT	CAC	CGC	TCG	GGC	TAG	AAG	GGG	TAG
3016	GCT	GAT	GTC	GGC	GAT	ATA	GGC	GCC	AGC	AAC	CGC	ACC	TGT	GCC	GCC
	CCA	CTA	CAG	CCG	CTA	TAT	CCG	CGG	TCG	TTC	GGG	TGG	ACA	CCG	CGG
3061	GCT	GAT	GCC	GGC	CAC	GAT	GGC	TCC	GGC	GTA	GAG	GAT	CCA	CAG	GAC
	CCA	CTA	CGG	CCG	GTG	CTA	CGC	AGG	CCG	CAT	CTC	CTA	GGT	GTC	CTG

Figure 9b (con't.)

22/27

3106	GGG	TGT	GGT	CCC	CAT	GAT	CGC	GTA	GTC	GAT	AGT	GGC	TCC	AAG	TAG
CCC	ACA	CCA	GCG	GTA	CTA	GGG	CAT	CAG	CTA	TCA	CCG	AGG	TTC	ATC	
3151	CGA	AGC	GAG	CAG	GAC	TGG	GCG	GCG	GCC	AAA	GCG	GTC	GGA	CAG	TGC
	GCT	TCG	CTC	GTC	CTG	ACC	CGC	CGC	CGG	TTT	CGC	CAG	CCT	GTC	ACG
3196	TCC	GAG	AAC	GGG	TGC	GCA	TAG	AAA	TTG	CAT	CAA	CGC	ATA	TAG	CGC
	AGG	CTC	TTG	CCC	ACG	CGT	ATC	TTT	AAC	GTA	GTC	GGG	TAT	ATC	GGC
3241	TAG	CAG	CAC	GCC	ATA	GTG	ACT	GGC	GAT	GCT	GTC	GGA	ATG	GAC	GAT
	ATC	GTC	GTG	GGG	TAT	CAC	TGA	CCG	CTA	CGA	CAG	CCT	TAC	CTG	CTA
3286	ATC	CCG	CAA	GAG	GCC	CGG	CAG	TAC	CGG	CAT	AAC	CAA	GCC	TAT	GCC
	TAG	GGC	GTT	CTC	CGG	GCC	GTC	ATG	GCC	GTA	TTG	GTT	CGG	ATA	CGG

Figure 9b (con't)

23/27

3331 TAC AGC ATC CAG GGT GAC GGT CCC GAG GAT GAC GAT GAG CGC CGC ATT  
ATG TCG TAG GTC CCA CTG CCA CGG CTC CTC CTC CTC CTC GCG TAA

3376 GTT AGA TTT CAT ACA CGG TGC CTG ACT GCG TTA GCA ATT TAA CTG  
CAA TCT AAA GTA TGT GCC ACG GAC TGA CGC ATT CGT TAA ATT GAC

3421 TGA TAA ACT ACC GCA TTA AAG CTT ATC GAT GAT AAG CTC TCA AAC  
ACT ATT TGA TGG CGT AAT TTC GAA TAG CTA CTC GAC AGT TTG

3466 ATG AGA ATT  
TAC TCT TAA

Total number of bases is: 3474.  
DNA sequence composition:  
2 OTHER;  
Sequence name: NPMTNFMPH.

Figure 9b (con't)

24/27

1 Met Val Arg Ser Ser Gln Asn Ser Ser Asp Lys Pro Val Ala  
16 His Val Val Ala Asn His Gln Val Glu Glu Gln Gly Ile His His  
31 His His His Val Asp Pro Gly Pro Met Ala Phe Arg Arg Pro  
46 Leu Glu Phe Pro Gly Gly Gln Gln His Ser Pro Gln Gly Tyr Gly  
61 Ser Gln Tyr Gly Gly Tyr Gly Gln Gly Gly Ala Pro Thr Gly Gly  
76 Phe Gly Ala Gln Pro Ser Pro Gln Ser Gly Pro Gln Gln Ser Ala  
91 Gln Gln Gln Gly Pro Ser Thr Pro Pro Thr Gly Phe Pro Ser Phe  
106 Ser Pro Pro Pro Asn Val Gly Gly Ser Asp Ser Gly Ser Ala  
121 Thr Ala Asn Tyr Ser Glu Gln Ala Gly Gly Gln Gln Ser Tyr Gly  
136 Gln Glu Pro Ser Ser Pro Ser Gly Pro Thr Pro Ala

Fig. 10

25/27

GGG CCC GAA CTT GAC GAA CTC GCC GTC GTA GCT GGC TTC CTC GTC	45
GGT CCA CAG CGC CCG CAT CGC TTC CAG GTA TTC GCG CAG CAT GGT	90
GCG GCG CCG GCC CGC CGG CAC GCC GTG GTC GGC GAG TTC GTC GGT	135
GTT CCA GCC GAA CCC GAC GCC GAG GCT GAC CCG GCC GGC GGA CAG	180
ATG GTC AAG GGT GGC AAT ACT TTT CGC CAG CGT GAT CGG GTC GTG	225
TTC GAC CGG CAG GGC CAC CGC GGT GGA CAG CCG CAC CCG CGA GGT	270
GAC GGC ACA GGC CGC GCC CAG ACT GAC CCA CGG GTC CAG GGT GCG	305
CAT GTA GCG GTC GTC GGG CAG CGA CGC GTC GCC GGT GGT CGG GTG	360
CGC GGC CTC CCG CTT GAT CGG GAT ATG CGT GTG TTC CGG CAC GTA	405
GAA GGT CGC AAA CCC GTG GTC GTC GGC AAG CTT CGC GGC CGC AGC	450
CGG AGA GAT GCC ACG GTC GCT GGT GAA AAG CAC AAG CCC GTA ATC	495
CAT GCA GTG AAT TAG AAC GTG TTC TAC CTC TGC GGG GCA AGC TGT	540
CGT GAT ACG GAC CGT CTC GCC GCG CGG TCG TCT GCG AAG CCC GCG	585
GCG AAG CCA ATG GCG ACG GCA CCG GCC GTC GCA CGT GCG CTA GCG	630
TGG GTG ATC GAC CGT GTC GCT CGC GCA GTG ACG CGC CTG CAA GCA	675
CCG CGT CGC ATC GCA ACC GTG GCG CCC GCT CGG CAC TAA AAG GCA	720
GTG GAA GCA ACA GGA GGA GCC ATG ACC TAC TCT CCC GGC AGC CCC	765
MET Thr Tyr Ser Pro Gly Ser Pro	
1	5

GGA TAT CCA CCG GCG CAG TCT GGC GGC ACC TAT GCA GGC GCC ACA	810
Gly Tyr Pro Pro Ala Gln Ser Gly Gly Thr Tyr Ala Gly Ala Thr	
10	15
15	20

CCA TCT TTC GCC AAA GAC GAC GAC GGC AAG AGC AAA CTC CCG CTC	855
Pro Ser Phe Ala Lys Asp Asp Asp Gly Lys Ser Lys Leu Pro Leu	
25	30
30	35

TAC CTC AAC ATC GCC GTG GTC GCC CTG GGT TTC GCG GGC GCC TAC CTG	900
Tyr Leu Asn Ile Ala Val Val Ala Leu Gly Phe Ala Ala Tyr Leu	
40	45
45	50

CTG AAT TTC GGC CCC ACC TTC ACC ATC GGC GGC GAC CTC GGC CCG	945
Leu Asn Phe Gly Pro Thr Phe Thr Ile Gly Ala Asp Leu Gly Pro	
55	60
60	65

Figure 11

GGT ATC GGC GGC CGC GCG GGT GAC GCC GGC ACC GCC GTC GTG GTG 990  
 Gly Ile Gly Gly Arg Ala Gly Asp Ala Gly Thr Ala Val Val Val  
 70 75 80

GCG CTG CTG GCC GCG CTG CTC GCC GGG CTG GGC CTG CTG CCC AAG 1035  
 Ala Leu Leu Ala Ala Leu Leu Ala Gly Leu Gly Leu Leu Pro Lys  
 85 90 95

GCC AAG AGT TAT GTG GGC GTG GTC GCG GTC GTC GCG GTG CTC GCC 1080  
 Ala Lys Ser Tyr Val Gly Val Val Ala Val Val Ala Val Leu Ala  
 100 105 110

GCG CTG CTG GCC ATC ACC GAG ACG ATC AAC CTG CCC GCC GGT TTC 1125  
 Ala Leu Leu Ala Ile Thr Glu Thr Ile Asn Leu Pro Ala Gly Phe  
 115 120 125

GCG ATC GGC TGG GCG ATG TGG CCG CTG GTG GCG TGC GTG GTG CTG 1170  
 Ala Ile Gly Trp Ala MET Trp Pro Leu Val Ala Cys Val Val Leu  
 130 135 140

CAG GCG ATC GCC GCG GTG GTC GTG GTC CTG CTG GAC GCC GGG GTG 1215  
 Gln Ala Ile Ala Ala Val Val Val Val Leu Leu Asp Ala Gly Val  
 145 150 155

ATC ACG GCG CCG GCG CCG CCC AAG TAC GAC CCC TAC GCG CAG 1260  
 Ile Thr Ala Pro Ala Pro Arg Pro Lys Tyr Asp Pro Tyr Ala Gln  
 160 165 170

TAC GGC CAA TAC GGG CAA TAC GGC CAG TAC GGG CAA CAG CCC TAC 1305  
 Tyr Gly Gln Tyr Gly Gln Tyr Gly Gln Gln Gln Pro Tyr  
 175 180 185

TAC GGT CAG CCG GGC GGT CAG CCC GGG GGC CAG CCG GGT GGT CAG 1350  
 Tyr Gly Gln Pro Gly Gly Gln Pro Gly Gly Gln Pro Gly Gly Gln  
 190 195 200

CAG CAT TCG CCG CAG GGC TAC GGG TCG CAG TAC GGC GGT TAC GGC 1395  
 Gln His Ser Pro Gln Gly Tyr Gly Ser Gln Tyr Gly Gly Tyr Gly  
 205 210 215

CAG GGC GGC GCT CCG ACC GGC GGT TTC GGT GCC CAG CCG TCG CCG 1140  
 Gln Gly Gly Ala Pro Thr Gly Gly Phe Gly Ala Gln Pro Ser Pro  
 220 225 230

CAG TCC GGC CCG CAA CAG TCC GCG CAG CAG CAG GGC CCG TCC ACA 1485  
 Gln Ser Gly Pro Gln Gln Ser Ala Gln Gln Gln Gly Pro Ser Thr  
 235 240 245

Figure 11 (cont'd 1)

~~27/27~~

CCG CCC ACC GGC TTC CCC AGC TTC AGC CCG CCG CCC AAC GTC GGC 1530  
Pro Pro Thr Gly Phe Pro Ser Phe Ser Pro Pro Pro Asn Val Gly  
250 255 260

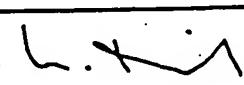
GGG GGA TCG GAC TCC GGT TCG GCG ACC GCC AAT TAC TCC GAG CAG 1575  
Gly Gly Ser Asp Ser Gly Ser Ala Thr Ala Asn Tyr Ser Glu Gln  
265 270 275

GCC GGT GGC CAG CAG TCC TAC GGC CAG GAG CCT TCT TCA CCG TCT 1620  
Ala Gly Gly Gln Gln Ser Tyr Gly Gln Glu Pro Ser Ser Pro Ser  
280 285 290

GGG CCG ACG CCC GCC TAA CGT GCC CTG TCG CGC CTA GTC GGG AAC 1665  
Gly Pro Thr Pro Ala ---  
295

GTG CCC CAG AGT GAC ACG ACG GGT GGA GGA CAA CCG GGC AGC GGG CGC 1710  
TCG CCA GGC GCG TGA CCT CGT CAG GGT CGC GTT CGC CCC GGC GGT 1755  
GGT GGC ACT GGT CAT CAT CGC CGC GGT CAC GCT GAT CCA GTT GTT 1800  
GAT CGC CAA CAG CGA CAT GAC CGG CGC GTT GGG GAA TTC 1839

Figure 11 (con't 2)

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.C1. 5 C12N15/31; C07K13/00;	G01N33/569; C12N15/62;	A61K39/04; C12N1/21;	A61K39/395 C12N5/10
<b>II. FIELDS SEARCHED</b>			
Minimum Documentation Searched <sup>7</sup>			
Classification System	Classification Symbols		
Int.C1. 5	C07K ;	C12P ;	C12Q ; A61K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>			
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>			
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	
A	WO,A,8 903 892 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 5 May 1989 see page 4, paragraph 2 see page 6, paragraph 4 - page 7, paragraph 1 ----	5	
A	WO,A,8 808 456 (J. HERMAN-TAYLOR & J.-J. MCFADDEN) 3 November 1988 see abstract see page 27, paragraph 2 ----	1,5, 17-24	
		-/-	
<p>* Special categories of cited documents:<sup>14</sup></p> <ul style="list-style-type: none"> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the International filing date</li> <li>"EL" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the International filing date but later than the priority date claimed</li> </ul> <p>"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>			
<b>IV. CERTIFICATION</b>			
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report		
15 MAY 1992	23.08.92		
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer THIELE U.H.-C.H. 		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF CLINICAL MICROBIOLOGY vol. 25, May 1987, WASHINGTON D.C., US pages 796 - 801; J. J. MCFADDEN: 'Crohn's Disease-Isolated Mycobacteria Are Identical to Mycobacterium paratuberculosis, as Determined by DNA Probes That Distinguish between Mycobacterial Species' see abstract see page 798, left column, paragraph 5 - right column, paragraph 1 see page 799, right column, line 37 - page 800, line 5	5

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. EP 9200661  
SA 57664

This annex lists the patent family members relating to the patent documents cited in the above-indicated International search report.  
The numbers are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for those particulars which are merely given for the purpose of information. 15/05/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8903892	05-05-89	US-A-	4918178	17-04-90
		AU-A-	2787889	23-05-89
WO-A-8808456	03-11-88	AU-A-	1628688	02-12-88
		EP-A-	0288306	26-10-88
		EP-A-	0356450	07-03-90
		JP-T-	3503837	29-08-91

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